The Role of $P2Y_{12}$ Receptor Antagonists in Thrombogenesis

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1 Zusammenfassung

1.1 Hintergrund

Der Adenosindiphosphat-Rezeptor $P2Y_{12}$ bewirkt Plättchenaggregation und trägt zur Bildung arterieller Thrombosen bei. $P2Y_{12}$-Antagonisten, wie Clopidogrel und Ticagrelor, werden daher zur Prävention von Herzinfarkten und Schlaganfällen in Patienten mit einem akuten Koronarsyndrom (AKS) verwendet. Ticagrelor war Clopidogrel in der Reduktion der Mortalität in diesen Patienten überlegen und die zugrunde liegenden Mechanismen sind nicht vollständig geklärt. Die arterielle Thrombose ist das zentrale Ereignis in der Entwicklung des AKS und das Gefässendothel ist wesentlich an dessen Entstehung beteiligt. Mögliche Effekte von $P2Y_{12}$-Antagonisten auf das Endothel und seinen prokoagulatorischen Faktoren, insbesondere dem Gewebefaktor, wurden bisher nicht untersucht. Patienten mit AKS haben häufig Komorbiditäten wie Vorhofflimmern (VHF) und benötigen daher eine zusätzliche antikoagulatorische Therapie zum Schutz vor Embolien kardialer Thromben aus dem linken Herzohr. Ähnlich dem Endothel, führt eine Aktivierung des Endokards zu einer Überexpression des Gewebefaktors sowie des Plasminogenaktivator-inhibitors-1, welche zur Thrombogenität in Patienten mit Vorhofflimmern beitragen. Ob bestimmte $P2Y_{12}$-Antagonisten antithrombotische Eigenschaften auf das Endokard linker Herzohren aufweisen und dadurch die Thrombogenität in Patienten mit VHF reduzieren könnten, wurde bisher nicht untersucht.

1.2 Material und Methoden

Menschliche aortale Endothelzellen wurden mit Ticagrelor oder mit dem aktiven Metaboliten von Clopidogrel behandelt und mit Tumor-Nekrose-Faktor-alpha (TNF-α) stimuliert. Anschliessend wurden die Expression und Aktivität des Gewebefaktors sowie die zugrunde liegenden molekularen Mechanismen erforscht. Zusätzlich wurden C57BL/6 Mäuse mit Ticagrelor oder Clopidogrel behandelt und die Expression des endothelialen Gewebefaktors sowie die Bildung arterieller Thrombosen nach photochemische Schädigung des Endothels

der Halsschlagadern untersucht. Schliesslich gewannen wir Endokardzellen aus linken Herzohren von 14 Patienten mit VHF, welche sich einer elektiven Herzoperation unterzogen. Diese wurden mit Ticagrelor oder mit dem aktiven Metaboliten von Clipidogrel behandelt und mit TNF-α stimuliert bevor die Expression und Aktivität des Gewebefaktors und des Plasminogenaktivator-inhibitors-1 analysiert wurden.

1.3 Ergebnisse

Ticagrelor, im Gegensatz zum aktiven Metaboliten von Clopidogrel, verringerte die TNF-α-induzierte Expression und Aktivität des Gewebefaktors durch proteasomale Degradierung unter Einbezug der Signalmoleküle Phosphoinositide-3-Kinase und p70s6 Kinase und unabhängig vom $P2Y_{12}$ Rezeptor sowie dem equilibrative nucleoside transporter 1 (ENT1). Wie in unseren *in vitro* Experimenten, reduzierte Ticagrelor, nicht aber Clopidogrel, die Expression des endothelialen Gewebefaktors in Mausarterien und verlängerte die Zeit bis zur Entstehung arterieller Thrombosen. Dabei waren die Plättchenhemmung, die Gewebefaktoraktivität im Plasma und die systemische Koagulation in beiden Gruppen vergleichbar. In Endokardzellen linker Herzohren von Patienten mit Vorhofflimmern verminderte Ticagrelor, nicht aber Clopidogrel, die TNF-α-induzierte Expression und Aktivität des Gewebefaktors und des Plasminogenaktivator-inhibitors-1.

1.4 Konklusionen

Ticagrelor, nicht aber Clopidogrel, weist lokale antithrombotische Eigenschaften auf das Endothel auf und reduziert die Entstehung von arteriellen Thrombosen im Vergleich zu Clopidogrel. Zudem zeigt Ticagrelor, nicht aber Clopidogrel, lokale antithrombotische Effekte im Endokard linker Herzohren von Patienten mit VHF auf. Die antithrombotischen Eigenschaften von Ticagrelor tragen möglicherweise zur Reduktion der Mortalität von Patienten mit AKS in klinischen Studien bei und vermindern allenfalls das Risiko für systemische Thromboembolien in Patienten mit Vorhofflimmern.

2 Summary

2.1 Background

The adenosine diphosphate receptor $P2Y_{12}$ mediates platelet aggregation and contributes to arterial thrombus formation; therefore, $P2Y_{12}$ antagonists, such as clopidogrel and ticagrelor, are used to prevent myocardial infarction and stroke in patients with acute coronary syndromes (ACS). Yet, ticagrelor was found superior over clopidogrel in decreasing mortality in these patients and the underlying mechanisms are not entirely understood. Arterial thrombosis is the crucial step in ACS and the endothelium plays a pivotal role in mediating thrombus formation. However, possible off-target effects of $P2Y_{12}$ antagonists on the endothelium and its key procoagulant factors such as tissue factor (TF), have not yet been investigated. Frequently, patients with ACS have comorbidities such as atrial fibrillation (AF) requiring anticoagulant therapy to prevent embolism of thrombi originating from left atrial appendages (LAA). Similar to endothelial cells, activation of LAA endocardial cells induces procoagulant TF and plasminogen activator inhibitor-1 (PAI-1) expression favouring thrombus formation in patients with AF. Whether certain $P2Y_{12}$ antagonists possess antithrombotic properties on endocardial cells and may reduce thrombogenicity in AF patients has not yet been investigated.

2.2 Material and methods

Human aortic endothelial cells (HAECs) were incubated with ticagrelor or clopidogrel active metabolite (CAM) before stimulation with tumor necrosis factor-alpha (TNF-α); next, TF protein expression and enzyme activity as well as the underlying molecular mechanisms were investigated. Additionally, C57BL/6 mice were treated with ticagrelor or clopidogrel before endothelial TF expression was determined in common carotid arteries and photochemical-induced arterial thrombosis was compared between the groups. Finally, endocardial cells were isolated from LAA of 14 patients with AF undergoing elective cardiac

surgery. Endocardial cells were treated with ticagrelor or CAM and stimulated with TNF-α and TF as well as PAI-1 expressions and enzyme activities were analysed.

2.3 Results

Ticagrelor, unlike CAM, decreased TNF-α-induced TF activity and expression via proteasomal degradation in HAECs. These effects were mediated via the signalling molecules phosphoinositide 3-kinase and p70s6 kinase and independently of the $P2Y_{12}$ receptor and the equilibrative nucleoside transporter 1 (ENT1). Likewise, ticagrelor, but not clopidogrel, reduced endothelial TF expression in common carotid arteries of C57BL/6 mice and prolonged time to arterial thrombosis; meanwhile, platelet inhibition, plasma TF activity and systemic coagulation were comparable between the two groups. In LAA endocardial cells from AF patients, ticagrelor, unlike CAM, decreased TNF-α-induced TF and PAI-1 protein expressions and enzyme activities.

2.4 Conclusions

Ticagrelor, but not clopidogrel, displays local antithrombotic properties on the endothelium and, compared with clopigorgel, reduces arterial thrombosis. Likewise, ticagrelor, but not CAM, exhibits local antithrombotic properties in AF patients by reducing expressions and activities of TF and PAI-1 in LAA endocardial cells. The specific antithrombotic properties of ticagrelor may contribute to the reduced mortality in ACS patients observed in clinical trials and may prevent systemic thromboembolism in patients with AF.

3 Introduction

3.1 Epidemiology of cardiovascular disease

Cardiovascular (CV) disease (CVD), including coronary heart disease and stroke, is the leading cause of death in Europe (Fig. 1)[1], the United States of America[2] and worldwide.[3] In Europe, 4 million deaths per year are attributed to CVD, thereby representing 45 % of all deaths[1]. Interestingly, absolute and relative numbers of CV mortality are greater in women than in men, which is due to a higher number of cerebrovascular and “other CVD”, whereas coronary heart disease is comparable among both sexes (Fig. 1).[1]

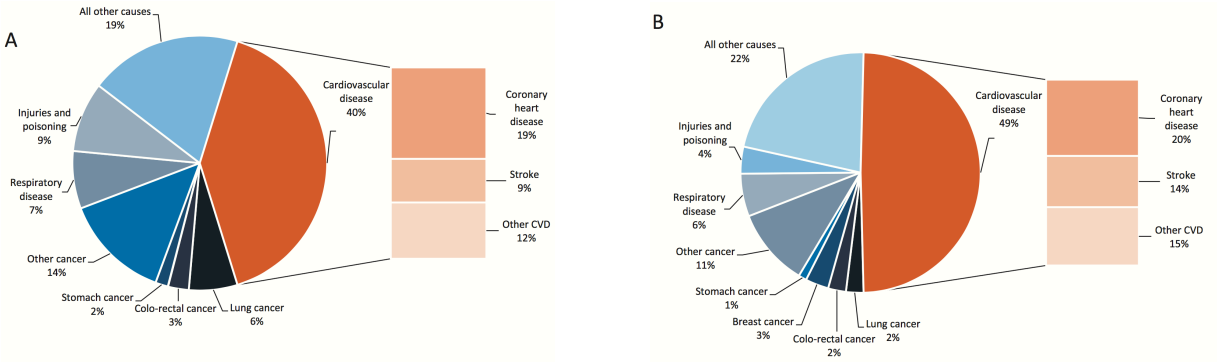

Figure 1. Deaths due to major causes in (A) men and (B) women in Europe. Source:[1]

From 1990 to 2013, CV mortality increased from 12.3 to 17.3 million deaths worldwide, representing 25.9 % and 31.5 % of all non-communicable diseases, respectively.[1] One explanation for this observation represents the ageing population[4] since age is a major risk factor for the development of CVD.[5] Ageing results from improved healthcare[6] and better lifestyle conditions[7] and is expected to continue in the near future causing substantial increase of disease burden and healthcare costs.[4] Besides coronary heart disease and stroke, atrial fibrillation represents the most common cardiac arrhythmia with a prevalence of 1.5 – 2 % in the general population and occurs at an average age of 75 – 85 years.[8] In addition to the increased risk for mortality, thromboembolic complication, such as stroke, cause a significant disease burden and healthcare costs, which are estimated to further increase in the next decades.[8]

3.2 Endothelial function and dysfunction

Vessel wall

The vascular wall consists of an inner layer of endothelial cells, followed by vascular smooth muscle cells (VSMC) and adventitial cells[9] (Fig. 2). The endothelial layer represents a roughly 0.2 μm thick monolayer covering the whole vasculature and thereby an area of approximately 3000 – 6000 m^2, whereas the majority of cells are considered microvascular endothelial cells covering particularly capillaries.[10] The volume of the entire endothelium is comparable to the volume of the liver.[11] The endothelium preserves blood fluidity by preventing thrombus formation, adjusts tissue perfusion by regulating vascular tone, regulates vascular permeability and inflammatory responses, and plays a pivotal role during angiogenesis.[11]

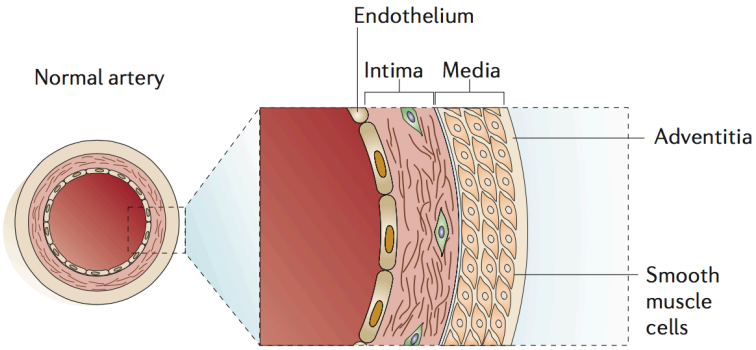

Figure 2. Arterial wall structure. The arterial wall is divided into the tunica intima containing ECs, the tunica media containing VSMCs and the tunica adventitia containing adventitial cells. EC = endothelial cell, VSMC = vascular smooth muscle cell. Source:[9]

Endothelial function

In healthy conditions, the endothelium prevents thrombus formation by physically separating platelets and coagulation factors from subendothelial prothrombotic mediators, such as collagen and tissue factor (TF)[12], and by expressing anticoagulant mediators such as nitric oxide (NO)[13,14], prostacyclin (PGI_2)[15], ectonucleotidase CD39[16], tissue factor pathway inhibitor (TFPI)[17] and antithrombin III[18] among others. During resting state, endothelial cells do not interact with leukocytes since expression of adhesion molecules is down regulated and

chemokines are not secreted.[19] Vascular tone is regulated via various vasoactive substances causing vasodilatation, such as NO, prostacyclin, adenosine and hydrogen peroxide (H_2O_2) or mediators causing vascular contraction, such as thromboxane A2, endothelin, angiotensin II, superoxide anion (O^2-) but also hydrogen peroxide.[11]

Endothelial dysfunction and reactive oxygen species

Endothelial dysfunction is characterised by reduced vasodilatatory properties or a shift toward a proinflammatory or a prothrombotic state[20]; it is associated with aging, diabetes, hypertension and atherosclerosis[11] and predicts CV outcome in patients with peripheral arterial disease[21], atherosclerosis[22], ACS[23] and heart failure.[24]

Despite the complexity of the pathogenic mechanisms leading to ED, oxidative stress is considered a central player in this respect.[11] Relevant reactive oxygen species (ROS) for vascular pathology include NO, superoxide anion, hydrogen peroxide and peroxynitrite ($ONOO^-$).[25] NO is produced by endothelial NO synthase[26] and by inducible NO synthase in endothelial cells, macrophages and VSMCs during inflammatory state.[27] It causes endothelial-dependent vasodilatation[13,14], inhibits platelet adhesion[28] and aggregation[29], and adhesion of leukocytes by inhibiting the expression of adhesion molecules.[20] Superoxide anion is produced by various enzymes including NO synthase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase[30] and occurs in all cell types. In the presence of superoxide anion, NO reacts to $ONOO^-$, which causes oxidation of proteins, deoxyribonucleic acids and lipids[31] leading to the formation of oxidized low-density lipoprotein (LDL)[25] among others. Alternatively, $O2^-$ is reduced to H_2O_2 by superoxide dismutase and finally dismutated to water and oxygen by catalase or glutathione peroxidase.[11] In the presence of copper and ion or superoxide however, H_2O_2 forms highly reactive hydroxyl radicals (OH).[11]

ROS affect endothelium-derived vasorelaxation by uncoupling NO synthase thus reducing the bioavailability of NO and by inhibiting the downstream NO target guanylyl cyclase.[11] Also, ROS cause vasoconstriction by multiple mechanisms including increased release of calcium from the sarcoplasmatic reticulum[32] and activation of cyclooxygenase-1 and subsequent production of endothelium-derived contracting factors.[33] Further, ROS induce the expression of endothelial adhesion molecules[34] and monocyte chemoattractant protein-1[35] enhancing leukocyte-endothelium interaction and thereby modulating inflammatory responses.

3.3 Atherosclerosis

Atherosclerosis may be defined as a thickening and loss of elasticity of arterial walls due to atherosclerotic plaque formation[36,37] typically affecting the subendothelial intima of large and medium-sized arteries.[38] Atherosclerotic plaques consist of connective tissue, such as collagen and proteoglycans, cholesterol and phospholipids as well as cellular components including macrophages/foam cells, T-lymphocytes and VSMC[36-38] (Fig. 3).

ED, a shift of the endothelium towards a prothrombotic and proinflammatory state with a reduced vasodilatory capacity, is driven by classical CV risk factors such as dyslipidaemia, diabetes and hypertension[11] and is considered a precursor of atherosclerosis.[39] ED causes increased permeability and leukocytes adhesion to the endothelium[40] (Fig 3 B). During the early phase of plaque formation, intimal thickening occurs due to accumulation of connective tissue, particularly proteoglycans, followed by lipid deposition[41] (Fig. 3C).

Lipoproteins containing apolipoprotein B, such as LDL and presumably lipoprotein(a), are of particular importance for plaque development. LDL accumulates in the subendothelial space through binding to extracellular connective tissue, i.e. proteoglycans.[42] Interaction of LDL with proteoglycans depends on LDL size and density, whereas small and dense particles possess higher binding capacities.[42] LDL retention increases susceptibility of multiple lipoprotein modifications such as self-aggregation, cleavage and oxidation, thereby transforming LDL

particles proatherogenic, finally, leading to the progression of atherosclerotic plaque formation.[42] Indeed, oxidized LDL has been demonstrated in human atherosclerotic plaques.[43] From a clinical perspective, plasma levels of LDL strongly correlate with the development of atherosclerosis.[44] Consistently, reduction of LDL has been shown to reduce the risk for CV events[45] strongly supporting a causal concept between LDL cholesterol and atherosclerosis development and progression.

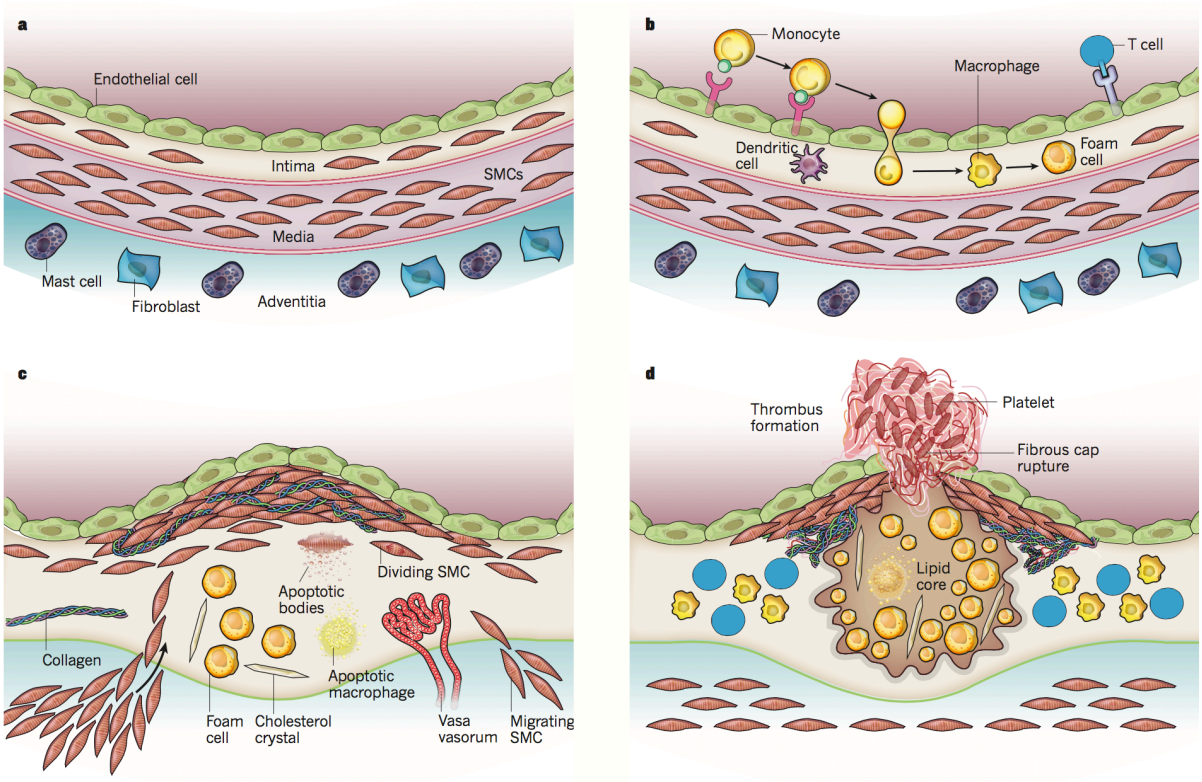

Figure 3. Atherosclerosis development. A) Healthy arterial wall. **B)** Initiation of atherosclerosis by transmigration of leukocytes and subsequent foam cell formation of monocytes through lipid uptake. **C)** Progression of atherosclerotic lesion through VSMC proliferation and migration, production of extracellular matrix connective tissue, apoptosis of macrophages and finally, accumulation of apoptotic bodies forming a necrotic core. **D)** Fibrous cap rupture and subsequent formation of an arterial thrombus through interaction of platelets and coagulation factors. Source: [36]

Endothelial transmigration of leukocytes including monocytes and T-lymphocytes (Fig. 3B) depends on the expression of endothelial adhesion molecules[40] and is mediated by chemoattractants[46,47], which can be stimulated by oxidized LDL.[46] Furthermore, both LDL

aggregation[48] and oxidation stimulate LDL uptake in macrophages[49] through scavenger receptors[50] finally leading to LDL degradation, intracellular lipid deposition and foam cell formation.[51] Scavenging of oxidized LDL through macrophages may reduces detrimental effects on surrounding cells, such as endothelial cells or VSMC; nevertheless, progressive lipid deposition magnifies inflammatory responses and overcomes such compensatory mechanisms of macrophages.[40] After adherence and migration, T-lymphocytes are in turn activated by macrophages through presentation of oxidized LDL[52] and secretion of cytokines including interferon-y and tumor necrosis factor-alpha (TNF-α).[53] Likewise, monocytes that transform to proinflammatory M2 macrophages secrete proinflammatory cytokines[54] thereby further enhancing the inflammatory response. Some foam cells undergo apoptosis followed by necrosis and release lipids, which together with necrotic cell components form the necrotic core in atherosclerotic plaques.[55]

In addition to monocytes and leukocytes, VSMC contribute to atherosclerotic plaque formation either by proliferation of VSMC in the intima, present in human arteries[56], or migration from the VSMC layer of the vessel[37] (Fig. 3C). Recently it has been proposed that in addition to local VSMC, bone marrow-derived stem cells contribute to atherosclerotic vascular pathology by homing, differentiation and migration.[57] VSMC secrete connective tissue like collagen and elastin, which together build the fibrous cap covering the atherosclerotic plaques and preventing plaque rupture[36] (Fig. 3D).

3.4 Tissue factor and initiation of the coagulation cascade

Tissue factor

TF is a transmembrane glycoprotein, which initiates the extrinsic coagulation cascade by binding coagulation factor VII[58], thereby activating factor IX and X[59] finally resulting in thrombin and subsequent fibrin formation.[60] Full length TF is a 47-kDa protein encoded by 6 exons located at chromosome 1 and comprises 263 amino acids, whereas the extracellular

domain consists of residues 1 – 219, the transmembrane domain of residues 220 – 242 and the cytoplasmic domain of residues 243 – 263.[58] The factor VII binding side is located at the extracellular domain.[61] In addition to full length TF, alternatively spliced TF has been described, which lacks exon 5.[62] Therefore, alternatively spliced tissue factor contains only 206 amino acids, whereas residues 1 – 166 are identical to full length TF[62] representing its extracellular domain and contain the factor VII binding side;[61] thus alternatively spliced TF is considered to be able to initiate coagulation.[62] The remaining residues 167 – 206 differ from full length TF due to a frameshift mutation and represent a unique C-terminus.[62] Since the transmembrane domain is not expressed, alternatively spliced TF is soluble and circulates in blood.[62] Possible sources of alternatively spliced TF include monocytes[62] and endothelial cells.[63] Alternatively spliced TF has been detected in human thrombi and is believed to contribute to thrombogenicity;[62] however, its procoagulant activity was not proven by others[64,65] and therefore its role in thrombosis is unclear. In addition to active cell surface TF, inactive and so called “encrypted” TF has been described.[66] Like active TF, it binds factor VII, however, does not activate factor X sufficiently to prompt procoagulant activity.[66] Transformation to the active form is mediated by protein-disulfide isomerase[67] and has been shown to be crucial for fibrin generation and thrombus formation in mice.[68]

TF is expressed in endothelial cells[69], VSMCs, the adventitia[70], monocytes[71], platelets and on circulating TF-containing microparticles released from platelets[72,73], endothelial cells and monocytes.[74] Furthermore it is expressed not only in the necrotic core of atherosclerotic plaques, but also in macrophages and the endothelium covering human atherosclerotic plaques.[70,75,76] In physiological conditions only spare amount of TF are found in endothelial cells; however, during pathological conditions, such as high shear flow as occurring in a stenosed vessel[77] and in the presence of various inflammatory cytokines such TNF-α and interleukin 1[78] as well as thrombin[79], CD40 ligand[80] and oxidized LDL[81], TF expression is

increased in endothelial cells. Likewise, interleukin 1 induces tissue factor expression in monocytes[82] and CD40 ligand in monocytes[83] and VSMCs.[84]

Initiation of the coagulation cascade

Tissue factor represents a cellular receptor for the plasma serine protease factor VII or the activated form, factor VII_a[85] (Fig. 4). Binding of TF enhances factor VII activation to VII_a[86] and binding of TF is essential to increases factor VII_a enzyme activity in order to activate factor IX and factor X to factor IX_a and X_a, respectively.[85] Activation of factor VII to factor VII_a is further mediated by factor IX_a and X_a[87] as well as factor VII_a itself.[88] Next, factor X_a forms the prothrombinase complex with factor V_a on TF-containing cells,[89] thereby transforming small amounts of prothrombin to thrombin, which subsequently amplifies the coagulation signal by activating factor V, VIII and XI on platelet surfaces[90] (Fig. 4). Finally, thrombin transforms fibrinogen to fibrin[91] and activates platelets by cleavage of protease-activated receptors.[92]

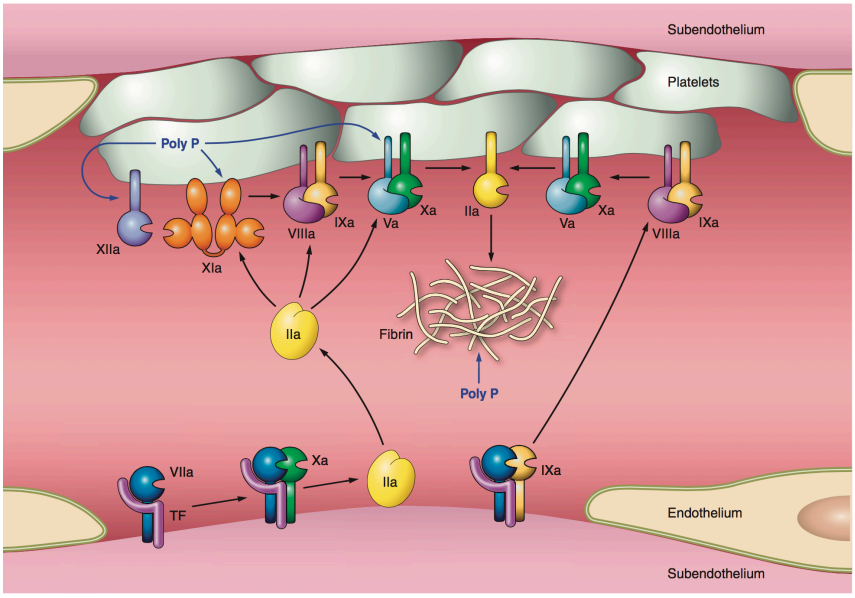

Figure 4. Coagulation system. TF binds activated factor VII (VII_a) and activates factor X and factor IX. Factor X_a leads to the production of small amounts of thrombin, which in turn activates factor V (cofactor of factor X), factor VIII (cofactor of factor IX) and factor XI. Factor XI_a further enhances factor IX activation. Factor IX_a together with cofactor $VIII_a$ (tenase complex) increase factor X activation. Finally, factor X_a binds factor V_a (prothrombinase complex) and increases thrombin production substantially. TF = tissue factor. Source:[86]

In order to preserve an antithrombotic endothelial cell surface in physiologic condition, TFPI counteracts TF-mediated activation of coagulation and subsequent thrombus formation[93]. TFPI is expressed in endothelial cells under quiescent conditions and, like TF, can be upregulated upon activation by inflammatory cytokines.[94] TFPI directly inhibits factor X_a,[93] most efficiently if factor X_a is integrated in the prothrombinase complex with factor V_a, calcium ions and phospholipids.[95] In addition, TFPI inhibits the TF/factor VII($_a$) through complexing with TF/factor VII($_a$) and X_a.[93]

Experimental and clinical studies

Experimental data revealed reduced arterial thrombus formation in low-TF expressing mice thus suggesting a pivotal role of vascular TF in thrombus formation.[96] Transgenic mice deficient in murine TF and expressing 1% of human tissue factor, revealed prolonged arterial occlusion times after photochemical injury of the carotid artery using bengal rose.[96] Neither did transplantation of low-TF bone marrow to wild-type animals increase, nor did transplantation of wild-type bone marrow to low-TF animals decrease arterial occlusion times confirming the relevance of vascular- rather than blood cell-derived TF in arterial thrombus formation.[96] In addition, anti-TF antibody treated rabbits displayed reduced arterial thrombus formation after injury and subsequent stenosis of the common carotid artery.[97] In clinical studies, increased levels of TF have not only been associated with cardiovascular risk factors including smoking[98], hypertension[99], diabetes[100] and dyslipidaemia[101] but also with acute coronary syndrome (ACS)[102] emphasizing the clinical relevance of TF.

3.5 Platelets and the $P2Y_{12}$ receptor

Platelets

Human platelets are anucleated blood cells derived from bone marrow magakaryocytes with an average life span of 9 to 10 days.[103] Despite the lack of DNA, platelets contain

megakaryocyte-derived messenger RNA[104] and are capable of protein synthesis.[105] One-hundred-fifty x 10^9 to 400 x 10^9 human platelets per litre of blood[103] are present in the circulation, which are in turn surrounded by 3000 – 6000 m^2 of endothelial surface.[10] In physiological conditions, the endothelium prevents platelet adhesion and aggregation by separating platelets from the subendothelial matrix, by inhibiting platelets through NO[13,14] and PGI_2[15] and by inhibiting platelet agonists through antithrombin III[18] and adenosine diphosphate (ADP) metabolism, among others.[106] During resting state platelets appear round/oval whereas upon endothelial damage, platelets adhere to the side of injury, become activated and aggregate, which results in profound shape changes such as cellular spreading.[12] Platelet are crucial to sustain haemostasis in case of injury; however, they may cause thrombus formation, subsequent vascular occlusion and tissue ischemia in pathological conditions.[12]

In case of vascular injury or endothelial disruption, subendothelial extracellular matrix containing different types of collagens, von Willebrand factor (vWF), fibronectin and laminin is exposed to the blood stream[107] (Fig. 5A). In high shear conditions as observed in the arterial circulation or in stenosed vessels, binding of the platelet receptor glycoprotein (GP) Ibα, part of the GPIb-V-IX complex, to extracellular vWF or plasma-derived vWF bound to subendothelial collagen, is crucial for platelet tethering.[108] Yet, additional interactions of platelet GPVI and/or integrin $\alpha_2\beta_1$ with collagen, platelet integrin $\alpha_5\beta_1$ with fibronectin or platelet integrin $\alpha_6\beta_1$ with laminin is required for platelet adhesion.[109] Adhesion of platelets to extracellular matrix proteins, particularly collagen and vWF, activates the platelet integrin $\alpha_{IIb}\beta_3$ receptor allowing binding of several ligands such as soluble vWF, fibrinogen and fibrin and by that cross-linkage of platelets, a key step in firm adhesion and platelet aggregation[12,107] (Fig. 5B). In parallel platelets produce thromboxane A2 and release dense granules containing ADP and α granules containing vWF, fibrinogen, fibronectin, p-selectin and interleukin 1 beta (Fig. 5A), which amplify platelet activation (ADP, thromboxane),

facilitate platelet-endothelium interaction (vWF, fibronectin), platelet-leukocyte interaction (fibronectin, fibrinogen p-selectin), leukocyte-endothelium interaction (p-selectin, interleukin 1 beta), platelet aggregation (vWF, fibrinogen) and finally, fibrin clot formation (fibrinogen, factor V).[12,109]

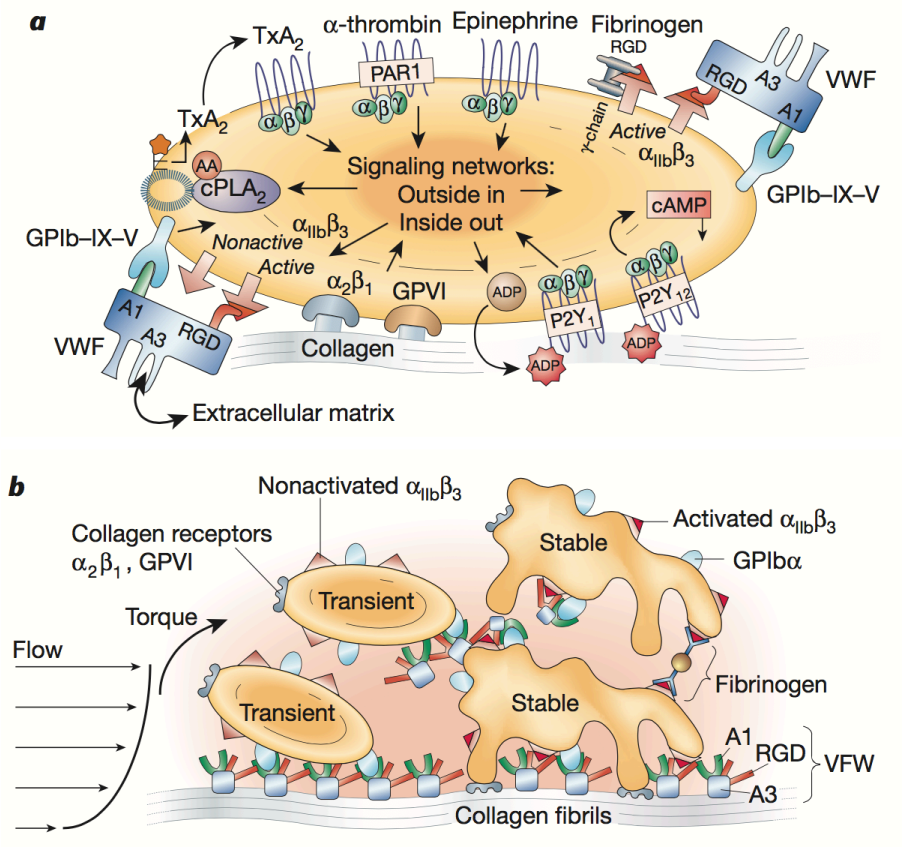

Figure 5. Platelet activation and aggregation. A) Platelet tethering to the vessel wall is mediated via GPIb-V-IX binding to subendothelial vWF. In addition, platelet GPVI and/or integrin $\alpha_2\beta_1$ binding to collagen is required for platelet adhesion and leads to activation of the $\alpha_{IIb}\beta_3$ receptor, thromboxane A2 production and release of dense and α granules containing ADP, vWF, fibrinogen, fibronectin, p-selectin and interleukin 1 beta further enhancing platelet activation and aggregation. In addition, platelets are activated by other agonists such as thrombin. B) Finally, activated $\alpha_{IIb}\beta_3$ receptor binds soluble vWF, fibrinogen and fibrin and mediates cross-linkage and thus, platelet aggregation. ADP = adenosine diphosphate, GP = glycoprotein, vWF = von Willebrand factor. Source:[12]

Adenosine diphosphate and the P2Y receptors

The released of ADP from dense granules, stimulated by collagen and thrombin,[110] causes significant amplification of platelet activation by auto- or paracrine binding to platelet purine

receptors $P2Y_1$[111] and $P2Y_{12}$.[112] Purine and pyrimidine receptors are divided in P1 receptors selective for adenosine and P2 receptors, which are further divided in the ligand-gated ion channels P2X receptors and the G protein-coupled P2Y receptors.[113] Binding of ADP to the metabotropic G_q-coupled $P2Y_1$ receptor results in phospholipase C activation, subsequent increase of inositol triphosphate and rise of cytosolic calcium from intracellular stores.[113] Instead, activation of the metabotropic G_i-coupled $P2Y_{12}$ receptor leads to an inhibition of the adenylyl cyclase and subsequent decrease of cyclic adenosine monophosphate.[113] In addition, the $P2Y_{12}$ receptor activates phosphoinositide 3-kinase (PI3K) and thereby the $\alpha_{IIb}ß_3$ receptor.[114] Activation of the $P2Y_1$ leads to platelet shape change and transient aggregation, whereas activation of the $P2Y_{12}$ receptor results in dense granule secretion and sustained platelet aggregation.[110] In summary, activation of both receptors is necessary to grant full platelet aggregation since blockade of each receptor individually reduces platelet aggregation.[115] Interestingly, inhibition of both receptors in parallel shows synergistic effects.[116]

3.6 $P2Y_{12}$ receptor antagonists

Due to the crucial role of platelets in arterial thrombus formation, platelet antagonists are routinely used in the clinic to reduce thrombogenesis and prevent cardiovascular complications such as myocardial infarction (MI) and stroke.[6] Beside from established platelet antagonists including the cyclooxygenase-1 antagonist aspirin[6], integrin $\alpha_{IIb}ß_3$ antagonists[117] and emerging inhibitors of GPIV[118], GPIb[119] and thromboxane A2 receptors[120], platelet ADP receptor $P2Y_{12}$ antagonists have been shown to reduce major adverse cardiac events in large clinical trials.[121-124] Currently four $P2Y_{12}$ receptor antagonists are approved for clinical use including clopidogrel, prasugrel, ticagrelor and cangrelor, whereas the first approved $P2Y_{12}$ receptor antagonist ticlopidine has been replaced due to adverse haematological side effects[125] and development of elinogrel was discontinued recently.[126] $P2Y_{12}$ receptor antagonists can be divided into thienopyridines including ticlopidine,

clopidogrel and prasugrel, in cyclopentyl-triazolo-pyrimidines including ticagrelor and in the adenosine triphosphate analogue cangrelor.[127]

The thienopyridines clopidogrel and prasugrel

Thienopyridines are indirect pro-drugs requiring conversion to the active metabolite by hepatic cytochrome P450 enzymes before irreversibly binding the $P2Y_{12}$ receptor.[127] Pharmacokinetic and pharmacodynamic properties differ significantly among $P2Y_{12}$ receptor antagonist. Clopidogrel not only exhibits weak inhibition of ADP-induced platelet aggregation with great inter-individual variability[128] but also 15 to 30 % of patients respond only marginally to clopidogrel treatment.[129] Importantly, higher platelet reactivity to ADP despite clopidogrel-treatment is associated with an increased risk of major adverse cardiac events.[129] Such non-responsiveness might be due to incompliance, differences in intestinal absorption and $P2Y_{12}$ receptor polymorphisms;[129] however, it has been proposed that variations in the cytochrome P450 3A4 considerably influences hepatic activation of clopidogrel.[130] Surprisingly, treatment adjustment in non-responders to clopidogrel did not improve cardiovascular outcome.[131]

Compared to clopidogrel, prasugrel shows a faster onset and higher level of platelet inhibition as well as a reduced inter-individual variability and lower non-responsiveness[132,133] due to a more efficient conversion of prasugrel to its active metabolite.[134] Consequently, prasugrel, compared with clopidogrel, reduced the primary composite endpoint, the rate of death from cardiovascular causes, non-fatal MI, or non-fatal stroke in ACS patients.[122] On the other hand, prasugrel increased major bleeding events and therefore, did not reduce overall mortality in these patients.[122]

The cyclopentyl-triazolo-pyrimidine ticagrelor

Ticagrelor and cangrelor are direct acting drugs, do not need hepatic transformation and bind to the $P2Y_{12}$ receptor reversibly.[127] Ticagrelor, similarly to prasugrel, exhibits a faster onset, a greater level of and a more rapid offset of inhibition of ADP-induced platelet aggregation.[135]

Due to reversible binding, ticagrelor may be more protective from platelet aggregation in patients with high platelet turnover due to a more sustained platelet inhibition of newly formed (reticulated) platelets.[136] Patients with higher number of reticulated platelets are at higher risk for cardiovascular events.[137] Correspondingly, aggregation of immature platelets correlated with prasugrel, but not ticagrelor-treated patients with ACS.[138] In large randomized clinical trials, ticagrelor proved higher efficacy, compared with clopidogrel in ACS patients; ticagrelor reduced the primary composite endpoint, death from vascular causes, MI and stroke without increasing the primary safety endpoint, major bleedings although fatal intracranial and non-intracranial bleeds were higher in ticagrelor-treated patients[121]. Importantly, ticagrelor, compared with clopidogrel, also reduced overall mortality.[121] Furthermore, in patients with prior MI, ticagrelor, compared with clopidogrel, reduced cardiovascular death, MI and stroke, but also increased major bleedings.[139]

Pleiotropic effects of $P2Y_{12}$ receptor antagonists

Thienopyridines were shown to have multiple pleiotropic effects.[140] In apolipoprotein E-deficient mice prone to atherosclerosis, clopidogrel treatment reduced atherosclerotic lesion size by roughly one third, compared to control animals.[141] Anti-inflammatory properties have been described for prasugrel; in a mouse model for endotoxic shock syndrome, prasugrel reduced platelet p-selectin expression and platelet-leukocyte interaction as well thromboxane 2 and TNF-α production.[142] Both, ticlopidine and clopidogrel were found to mediate endothelial-dependent vasodilatation in pre-contracted arteries of rats.[143] In patients with coronary artery disease and peripheral endothelial dysfunction, single dosages of clopidogrel dose-dependently reduced endothelial dysfunction as assessed by flow-mediated dilatation of the brachial artery.[144]

Like for thienopyridines, pleiotropic effects have been described also for ticagrelor.[140,145] In addition to the $P2Y_{12}$ receptor, ticagrelor, but not thienopyridines or cangrelor, binds to the equilibrative nucleoside transporter 1 (ENT1),[146] which is expressed in red blood cells among

many others,[147] and reduces adenosine uptake.[146] Consequently, ticagrelor decreased adenosine uptake in human red blood cells[148] and increased plasma levels of adenosine in ACS patients treated with ticagrelor.[149] Adenosine inhibits platelet activation[150] and was found to contribute to platelet inhibition in human whole blood treated with ticagrelor.[151] Furthermore, ticagrelor, but not prasugrel or clopidogrel, reduced VSMC contraction in rats;[152] Treatment with ticagrelor revealed anti-inflammatory properties by reducing pulmonary oedema, neutrophil recruitment and lung damage in mice exposed to experimental abdominal sepsis.[153] In addition, ticagrelor, unlike clopidogrel, reduced MI in rats[154] and in pigs in an adenosine-dependant manner.[155] In patients with prior ACS, ticagrelor, compared with clopidogrel and prasugrel, improved peripheral arterial function after forearm ischemia.[156] Such additional pleiotropic effects may in part contribute to the differences in outcomes observed in randomized controlled trials comparing $P2Y_{12}$ inhibitors. [121,139]

3.7 Atherothrombosis

Arterial thrombosis on top of an atherosclerotic lesion is the key event in ACS and results from interactions between the vessel wall, blood cells including platelets, red blood cells and leukocytes and the coagulation system.[86,157-160]

Atherosclerotic plaques

Complex atherosclerotic lesions prone to rupture and to cause arterial thrombosis usually present a thin fibrous caps, are large in size and cause a small luminal area;[161] they typically cause expansive vascular remodelling and exhibit low calcification.[162] Furthermore, they contain a large lipid core, numerous leukocytes and fewer VSMC.[163]

During the early stage of plaque development, atherosclerosis causes vascular enlargement by expansive growth, which compensates vascular stenosis through the newly formed plaque; therefore, stenosis occurs late during plaque development.[164] Plaque rupture rather

than superficial erosions of the fibrous caps overlying the lipid or so-called necrotic core, causes the majority of fatal coronary thrombosis.[165]

Plaque rupture is associated with thin fibrous caps[166] and its stability is believed to be mediated primarily through collagen, which is synthetized by VSMCs and degraded by some matrix-metalloproteinases.[159] Accumulation of T-lymphocytes and macrophages, however, reduces collagen synthesis by inhibiting VSMCs and increase collagen breakdown by producing matrix-metalloproteinases, respectively.[159] In contrast to plaque rupture, plaque erosion is believed to be triggered primarily by endothelial apoptosis due to increased oxidative stress from activated leukocytes, which also increases up-regulation of pro-coagulant TF.[159] Interestingly, statin therapy not only reduces plasma low-density lipoprotein and lipid content of atherosclerotic plaques but also decreases atherosclerotic plaque macrophage activity, which may contribute to its well-defined cardiovascular protective effects.[167] Also, anti-inflammatory therapy with low-dose colchicine reduced cardiovascular events in patients with stable coronary artery disease[168] highlighting the importance of inflammation in atherosclerosis and subsequent arterial thrombosis.

Platelet adhesion, activation and recruitment

Upon plaque erosion or rupture due to underlying inflammatory mechanisms[159], subendothelial extracellular matrix is exposed. In high shear conditions, as occurring in the arterial vasculature, vWF is essential for platelet tethering[108] and subsequent binding of various platelet receptors to subendothelial collagen, fibrinogen and laminin enables platelet adhesion[109]. Platelet adhesion triggers release of platelet granules containing ADP and thromboxane, which enhances platelet activation.[12,109] Next, integrin $\alpha_{IIb}\beta_3$ receptors become activated and, together with platelet GPIb, binds vWF and other soluble molecules, whereby additional platelets are recruited and aggregate at the side of plaque erosion or rupture.[12,107]

Initiation, amplification and propagation of the coagulation cascade

In parallel to platelet recruitment, the coagulation cascade is initiated by TF.[157] Exposure of TF and subsequent binding of factor VII/factor VII_a[85] activates factor IX and factor X.[85] Next, factor X_a forms the prothrombinase complex with factor V_a on TF-containing containing cells[89], which leads to the formation of small amounts of thrombin.[91]

Following initiation of the coagulation cascade, thrombin not only leads to platelet activation via cleavage of protease-activated receptors 1 and 4[92] but also activates co-factor V, co-factor VIII and factor XI on platelet surfaces thereby amplifying the procoagulant signal.[90] During the propagation phase, large amounts of thrombin are produced.[90] After thrombin-mediated activation of factor XI to XI_a, factor XI_a activates factor IX to IX_a, which binds factor $VIII_a$ and thus forms the tenase complex activating factor X to X_a; factor X_a complexes with V_a and increases thrombin.[86] Finally, thrombin transforms fibrinogen to fibrin, which subsequently polymerizes to fibrin strands.[91] Lastly, after activation by thrombin among others, factor $XIII_a$ stabilizes polymerized fibrin strands to form a stable platelet clot.[169]

3.8 State of research in the field

In addition to aspirin, $P2Y_{12}$ receptor antagonists including clopidogrel, prasugrel and ticagrelor are crucial to reduce MI, stroke and CV death in patients suffering an ACS as shown in large randomized controlled trials.[121-123] Both prasugrel and ticagrelor exhibit greater inhibition of ADP-induced platelet aggregation, as compared with clopidogrel.[132,135] Yet, despite comparable efficacy in platelet inhibition[170] only ticagrelor, but not prasugrel, reduced overall mortality in ACS patients, as compared with clopidogrel.[121,122] Recently described pleiotropic anti-inflammatory[153] and vasodilative effects[152] of ticagrelor as well as the ability to inhibit the adenosine transporter ENT1, may in part explain this observation. Nevertheless, $P2Y_{12}$ receptors are not only expressed in platelets but also in the vessel

wall[171] and platelet-independent vascular effects of $P2Y_{12}$ antagonists on arterial thrombogenesis remain unknown.

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5 Original Articles

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Ticagrelor, but not Clopidogrel, Reduces Arterial Thrombosis via Endothelial Tissue Factor Suppression

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Abstract

Aims

The $P2Y_{12}$ antagonist ticagrelor reduces mortality in patients with acute coronary syndrome (ACS), compared with clopidogrel, and the mechanisms underlying this effect are not clearly understood. Arterial thrombosis is the key event in ACS; however, direct vascular effects of either ticagrelor or clopidogrel with focus on arterial thrombosis and its key trigger tissue factor have not been previously investigated.

Methods and Results

Human aortic endothelial cells were treated with ticagrelor or clopidogrel active metabolite (CAM) and stimulated with tumor necrosis factor-alpha (TNF-α); effects on procoagulant tissue factor (TF) expression and activity, its counter-player TF pathway inhibitor (TFPI) and the underlying mechanisms were determined. Further, arterial thrombosis by photochemical injury of the common carotid artery, and TF expression in the murine endothelium were examined in C57BL/6 mice treated with ticagrelor or clopidogrel. Ticagrelor, but not CAM, reduced TNF-α-induced TF expression via proteasomal degradation and TF activity, independently of the $P2Y_{12}$ receptor and the equilibrative nucleoside transporter 1 (ENT1), an additional target of ticagrelor. In C57BL/6 mice, ticagrelor prolonged time to arterial occlusion, compared with clopidogrel, despite comparable antiplatelet effects. In line with our *in vitro* results, ticagrelor, but not clopidogrel, reduced TF expression in the endothelium of murine arteries.

Conclusion

Ticagrelor, unlike clopidogrel, exhibits endothelial-specific antithrombotic properties and blunts arterial thrombus formation. The additional antithrombotic properties displayed by ticagrelor may explain its greater efficacy in reducing thrombotic events in clinical trials. These findings may provide the basis for new indications for ticagrelor.

Introduction

Cardiovascular disease is the leading cause of death worldwide[1]. Platelets play a crucial role in arterial thrombus formation, the key event in cardiovascular complications, such as myocardial infarction and stroke. Consequently, platelet antagonists targeting the adenosine diphosphate (ADP) receptor $P2Y_{12}$ (i.e. clopidogrel, prasugrel, or ticagrelor) in combination with acetylsalicylic acid is the treatment of choice for patients suffering from acute coronary syndrome (ACS) and for secondary prevention after stent implantation or coronary artery bypass grafting[2]. In clinical trials ticagrelor and prasugrel proved to be superior over clopidogrel[3,4]; in particular, ticagrelor reduced the composite end point mortality due to vascular causes, myocardial infarction or stroke in addition to overall mortality in patients with ACS, compared with clopidogrel[3]. The cyclopentyl-triazolo-pyrimidine ticagrelor displays a different pharmacokinetic and pharmacodynamic profile, compared with the thienopyridines clopidogrel and prasugrel. Specifically, ticagrelor is direct acting and reversibly binding the $P2Y_{12}$ receptor, whereas the thienopyridines are pro-drugs requiring hepatic metabolism to generate an irreversibly binding active metabolite. Higher levels of platelet inhibition complemented by an additional mechanism of action, the inhibition of cellular adenosine uptake via the equilibrative nucleoside transporter 1 (ENT1)[5-8] leading to increased adenosine plasma levels[9], may in part account for the superior effects of ticagrelor.

A growing body of evidence suggests off-target effects of $P2Y_{12}$ antagonists; however, effects of ticagrelor and clopidogrel on the endothelium, their underlying molecular mechanisms and potential effects on arterial thrombosis remain elusive.

Tissue factor (TF) is the key trigger of the extrinsic coagulation cascade[10] and plays a major role in the development of arterial thrombotic complications such as myocardial infarction and stroke[11]. Correspondingly, anti-TF antibody treatment blocks arterial thrombus formation[12]. Increased levels of TF have been observed in patients with classical cardiovascular risk factors, such as hypertension[13], diabetes[14] and dyslipidaemia[15], as well as in ACS[16]. TF is

expressed in various cell types including endothelial cells and different cytokines such as tumor necrosis factor-alpha (TNF-α) are known to induce TF expression and activity[17]. Correspondingly, elevated levels of TNF-α have also been reported in patients with ACS[18] and atrial fibrillation[19] and are further associated with cardiovascular disease progression and severity[20].

In the present study, we investigate pleiotropic effects of ticagrelor and clopidogrel on TNF-α-stimulated primary human aortic endothelial cells (HAECs), assess the underlying mechanisms and their potential relevance in a mouse model of photochemical injury-induced arterial thrombosis.

Methods

Drugs

Clopidogrel active metabolite (CAM) and ticagrelor were provided by Sanofi-Aventis, Germany and AstraZeneca, Mölndal, Sweden, respectively. Clopidogrel was purchased from Santa Cruz Biotechnology.

Animals and treatment

Twelve-week-old bodyweight-matched male C57BL/6 mice were treated with clopidogrel (48 mg/kg/day or 32 mg/kg/day) or ticagrelor (120 mg/kg/day or 80 mg/kg/day) given in the chow (clopidogrel, 0.06% w/w or 0.04 w/w; ticagrelor, 0.15% w/w or 0.1% w/w), for 2 weeks to evaluate dosages that provide full and comparable platelet inhibition in mice. Dosages of clopidogrel and ticagrelor differed 2.5 fold corresponding to dosages used in humans[2] and dosages known to provide comparable platelet inhibitory effects in rodents[21]. Experimental procedures involving mice were reviewed and approved by the institutional animal care committee (licence number TVA 165/2013; Kommission für Tierversuche des Kantons Zürich, Zurich, Switzerland).

Plasma concentrations of ticagrelor

In addition to pharmcodynamic evaluation of $P2Y_{12}$ receptor antagonist treatment, ticagrelor plasma concentrations were determined by protein precipitation and liquid chromatography mass spectrometry as described previously[22].

Whole blood aggregometry

Sodium-citrate (3.8%) anticoagulated blood was collected by cardiac puncture using a 24-gauge needle after euthanasia of mice by isoflurane. Finally, whole blood (diluted 1 to 3 in 0.9% saline) aggregometry was performed in response to ADP (10 μM; 384, chrono-log),

thrombin (1 U/mL; T7009, Sigma Aldrich) and collagen (10µg/mL; Kollagenreagens Horm, Takeda) by impedance aggregometer (Chrono-Log).

Arterial thrombosis

After 2 weeks of treatment with ticagrelor, clopidogrel or control chow, C57BL6 mice were exposed to photochemical injury of the common carotid artery (CCA) as previously described[23]. Briefly, mice were anaesthetized using pentobarbital (87 mg/kg body weight); after midline neck incision, the right CCA was exposed under an operating microscope. To induce photochemical injury of the endothelium, bengal rose (50 mg/kg body weight) was injected into the tail vein and the CCA was exposed to a laser light beam (1.5 mW, 540nm, Mellesgriot Inc.) up to 120 minutes. Blood flow and heart rate were monitored (Doppler flow probe carotid artery Transonic Systems Inc., 0.5VB) until occlusion occurred or for a maximum of 120 minutes, in case arterial thrombosis was not detected.

Plasma thrombin generation

Plasma thrombin generation was assessed by calibrated automated thrombogram as previously described[24]. Sodium-citrate (3.8%) anticoagulated murine whole blood was drawn by cardiac puncture and centrifuged (4'000 g, 10 min, 4°C) to receive platelet-poor plasma, which was then mixed with either PPP-Reagent (Thrombinoscope BV) containing TF (f.c. 6 pM) and phospholipids (f.c. 4.8 µM), or thrombin calibrator (Thrombinoscope BV). Next, fluorogenic thrombin substrate (Thrombinoscope BV), Fluo-Buffer (Hepes buffer, pH 7.35, 20 mM Hepes, with BSA 60 mg/ml, Thrombinoscope BV) and $CaCl_2$ (246 mM) were added. Thrombin generation was measured over time by Fluoroskan® Ascent reader (Thermo Labsystems) and thrombin generation curve was calculated by Thrombinoscope software (Thrombinoscope BV) to finally receive endogenous thrombin potential (ETP) (nmol thrombin x min).

Endothelial tissue factor expression

CCAs of mice were embedded in Optimal Cutting Temperature medium, snap frozen and cryosectioned. Sections were incubated with the primary antibodies rat anti-murine CD31 (BD 553370, 1:2500), used as an endothelial cell marker, and rabbit anti-TF (R8084[25], 1:200) for 45 min at room temperature. After washing, the secondary antibodies donkey anti-rat Cy3 (Jackson 712-166-153, 1:250) and donkey anti-rabbit Alexa488 (Jackson 711-545-15, 1:250) were applied for 45 min at room temperature. DNA was counterstained using UltraCruz Hard-set Mounting medium containing 4',6-diamidino-2-phenylindole (Santa Cruz, sc-359850). Images were acquired using a Zeiss Axiovert inverted widefield fluorescence microscope with Axiovision software. Composite images were generated using Photoshop (CC2015, Adobe) and endothelial TF mean fluorescent intensity was quantified and normalized to endothelial cell area.

Isolation of human platelets

Human platelets were used as positive control of $P2Y_{12}$ receptor mRNA and protein expression. Venous blood was drawn from a healthy volunteer not receiving any medication for 10 days and collected in sodium-citrate (3.8%) tubes (Becton Dickinson). Blood was centrifuged twice at 100 g for 10 minutes at room temperature to obtain platelet-rich plasma, which was centrifuged at 400 g for 15 minutes at room temperature. Finally, the platelet pellet was washed with 1 mL phosphate-buffered saline and after a final centrifugation step (400 g, 10 min, room temperature) exposed to either protein lysis buffer or TRIzol for Western blot or real-time polymerase chain reaction (RT-PCR), respectively.

Cell culture experiments

HAECs (Lonza) were used for experiments between passage 5 and 8, derived from 4 individual batches. Endothelial cells were cultured in endothelial growth basal medium-2, supplemented with endothelial growth basal medium-2 bullet kit (Lonza) and 10% fetal

bovine serum. After 24 hours of growth, cells underwent starvation for 24 hours using endothelial basal medium (Lonza) supplied with 0.5% fetal bovine serum. Cells were treated with concentration ranges of ticagrelor (10^{-7}, 10^{-6}, 10^{-5} M)[26] or CAM (1.5 x 10^{-8}, 1.5 x 10^{-7}, 1.5 x 10^{-6} M), which are in line with plasma concentrations found in humans[26], and stimulated with TNF-α (10 ng/mL) for various time points. Drugs were dissolved in dimethyl sulfoxide (f.c. 0.1%). Correspondingly, unstimulated cells and TNF-α-stimulated cells were treated with dimethyl sulfoxide (f.c. 0.1%) to exclude vehicle-dependent effects.

Western blotting

Protein expression was determined by Western blot analysis. Endothelial cells and human platelets were incubated with lysis buffer (NaCl 150 mM, EDTA 1 mM, NaF 1 mM,DTT 1 mM, aprotinin 10 mg/mL, leupeptin 10 mg/mL, $Na_3VO_4$0.1 mM, PMSF 1 mM, and NP-40 0.5%); protein concentration was determined, according to the manufacturer's recommendations (Bio-Rad); 20 – 30 μg of protein lysates were separated on an 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred to a polyvinylidene fluoride membrane by semi-dry transfer. Membranes were cut, according to the size of proteins of interest and incubated with primary antibodies overnight at 4°C on a shaker. Secondary antibodies were applied for 1 hour at room temperature. Densitometric analyses were performed and protein expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Antibodies against TF (ADG4507 and 4503; 1:2000) and tissue factor pathway inhibitor (TFPI) (ADG72; 1:8000) were purchased from American Diagnostica; anti-$P2Y_{12}$ (ab86195; 1:2000) antibody from Abcam; anti-GAPDH antibody (MAB374; 1:40000) from Merck Millipore. Secondary anti-mouse (1031-05) and anti-rabbit (4050-05) antibodies were obtained from SouthernBiotech; recombinant human TNF-α (210-TA) was purchased from R&D Systems; actinomycin D (A1410), MG132 (C2211), rapamycin (R0395), wortmannin (W1628), SB203580 (S8307), SP600125 (S5567), dipyridamole (D9766), adenosine receptor antagonist against A1 (8-cyclopentyl-1,3-dipropylxanthine), A2a (SCH

442416), A2b (MRS 1754) and A3 (VUF 5574) as well as adenosine (A4036) from Sigma Aldrich; cycloheximide (239765) from Merck Millipore; PD 98059 (9900) from Cell Signaling Technology.

Real-time PCR

Total RNA was extracted from HAECs or human platelets using TRI reagent (Sigma Aldrich), according to the manufacturer's recommendations. Conversion of the total cellular RNA to cDNA was performed with Moloney murine leukemia virus reverse transcriptase and random hexamers (GE Healthcare) in a final volume of 35 µl, using 2 µg of total RNA, according to manufacturer's recommendations. RT-PCR was performed in a QuantStudio 7 Flex RT-PCR cycler (Applied Biosystems), according to the manufacturer's instructions. All RT-PCR experiments were performed using the SYBR Select Master Mix provided by Applied Biosystems (Life Technologies). Each reaction (20 µl) contained 2 µl cDNA, 400 fmol of each primer and 10 µl of Master Mix. The following primers were used: $P2Y_{12}$ (1), $P2Y_{12}$ (2), TF, human L28 and human β-actin (ACTB). The amplification program consisted of 1 cycle at 95˚C for 10 min, followed by 40 cycles with a denaturing phase at 95˚C for 15 s, an annealing/elongation phase at 60˚C for 1 min. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. For verification of the correct amplification, PCR products were analyzed on an ethidium bromide stained 1.5% agarose gel. Cycle threshold (C_T) values for each gene were obtained for each sample and differences in C_T values between a test gene and endogenous controls (ΔC_T) were calculated and used for statistical analyses.

For quantitative RT-PCR the following primers were used; for human $P2Y_{12}$: sense primer (1) 5'-CTTTCTCATGTCCAGGGT-3', antisense primer (1) 5'-GTTGCCAAACCTCTTTGT-3'; sense primer (2) 5'-TTTCTCATGTCCAGGGTC-3', antisense primer (2) 5'-CTGCAGAGTGGCATCTGG-3; for human TF: sense primer: 5'-CCAAACCCGTCAATCAAGTC-3', antisense primer: 5'-TGCCAAGTACGTCTGCTTCA-3'; for

human L28: sense primer: 5'-GCATCTGCAATGGATGGT-3', antisense primer: 5'-TGTTCTTGCGGATCATGTGT-3'; for ACTB: sense primer: 5'- GCACAGAGCCTCGCCTT -3', antisense primer: 5'-GTTGTCGACGACGAGCG-3'. All primers were ordered from Microsynth.

Tissue factor activity assay

TF activity was determined as previously described[23] in cell lysates of HAECs and murine plasma by ELISA, according to the manufacturer's recommendations (Sekisui Diagnostics, ACTICHROME® TF, 846). Endothelial cells were lysed (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1% Triton X-100, pH 7.4), diluted 1:15 in assay buffer, and mixed with human factor VIIa and X, which leads to conversion of factor X to Xa; factor Xa subsequently cleaves the chromogenic substrate SPECTROZYME® FXa. Finally, absorbance was measured at 405 nm and after background subtraction, optical density was normalized to protein concentration as determined by Nanodrop 2000 Spectrophotometer (Thermo Scientific). Sodium-citrate (3.8%) anticoagulated plasma was mixed with factor VIIa and X and optical density of cleaved SPECTROZYME® FXa was determined at 490 nm and subtracted from absorbance at 405 nm. Finally, plasma TF (pM) was calculated, according to a standard curve.

Tissue factor mRNA stability

Stability of TF mRNA was investigated as previously described[27]. Endothelial cells were stimulated with TNF-α for 1 h to induce TF gene expression and transcription was subsequently terminated using actinomycin D (10 μg /mL). Next, cells were incubated with ticagrelor (10^{-5} M) for 1, 2, or 3 hours before cells were lysed with TRIzol. Messenger RNA was isolated and RT-PCR was performed. Values were plotted as percent of time 0 against time (min) and half-life was calculated by non-linear regression using Prism 6 (GraphPad software).

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA with *Tukey post hoc* test or unpaired two-tailed Student's *t*-test as appropriate. A probability value below or equal to 0.05 was considered as statistically significant and calculated by Prism 6 (GraphPad software).

Results

Ticagrelor, but not CAM, reduces TF expression and activity

Ticagrelor concentration-dependently decreased TNF-α-induced TF expression in HAECs (Fig. 1A), whereas no effect was observed with increasing concentrations of CAM (Fig. 1B). In addition to protein expression, TF activity was reduced by ticagrelor, compared with vehicle-treated cells (Fig. 1C). On the other hand, protein levels of TFPI were not affected by ticagrelor or CAM (Supplementary Fig. 1). Preincubation with both the PI3 kinase inhibitor wortmannin and the mTOR inhibitor rapamycin partially reversed ticagrelor-mediated TF reduction (Fig. 1D and 1E), whereas co-incubation with both kinase inhibitors abolished the effect of ticagrelor (Fig. 1F). Preincubation using the MAP kinase inhibitors for JNK (SP600125, 10^{-6} M), ERK (PD98059, 10^{-7} M) or p38 (SB203580, 10^{-6} M) did not reverse ticagrelor-mediated reduction of TF expression (data not shown).

Ticagrelor decreases endothelial TF by proteasomal degradation

To investigate whether the reduction of TF protein expression was due to a decrease in TF gene expression, RT-PCR was performed. Contrary to protein expression, mRNA levels were increased by ticagrelor, compared with vehicle-treated cells (Fig. 2A), suggesting a compensatory mechanism for the decrease of tissue factor protein expression. Additionally, potential alterations of mRNA half-life were examined. To this end, actinomycin D was added to stop transcription 1 hour after TNF-α stimulation. Next, cells were incubated with ticagrelor or vehicle for an additional 1, 2, or 3 hours. No differences in mRNA levels between the groups were observed at any time point examined (Fig. 2B). To further evaluate the underlying mechanism on TF reduction, post-translational mechanisms were investigated, as previously described[28]. HAECs were stimulated with TNF-α for 3 hours, after which TF protein expression has been reported to reach a steady state[28]. Next, translation was arrested with cycloheximide and simultaneously, cells were incubated with ticagrelor or

vehicle for additional 2 hours. TF protein expression was not altered by cycloheximide, compared with TNF-α. However, ticagrelor significantly reduced TF protein expression, compared with vehicle-treated cells (Fig. 3A) suggesting that ticagrelor reduced TF protein stability. In line with this, the proteasome inhibitor MG-132 rescued the effect of ticagrelor on TF reduction (Fig. 3B) suggesting that ticagrelor reduces TF by proteasomal degradation.

Effects of ticagrelor are mediated independently of $P2Y_{12}$ or ENT1

To investigate the presence of $P2Y_{12}$ receptor in various endothelial cells, RT-PCR as well as Western blot experiments were performed using human platelets as positive control. Interestingly, $P2Y_{12}$ mRNA was not detected in human endothelial cells of aortic, cerebral or cardiac origin by using two different pairs of primers for human $P2Y_{12}$, whereas it was clearly found in human platelets (Fig. 4A). Next, expression of $P2Y_{12}$ protein was investigated, which was detected in human platelets, but, excluding unspecific bands, could not be detected in any types of endothelial cells (Fig. 4B). The absence of $P2Y_{12}$ mRNA and protein suggests that the observed effects of ticagrelor were mediated independently of the $P2Y_{12}$ receptor.

Next, we investigated whether the observed effects of ticagrelor were mediated by ENT1 inhibition and subsequent increase of adenosine, as recently discussed[8,21]. Therefore, TNF-α-stimulated cells were treated with increasing concentrations of adenosine to mimic the effects of ticagrelor. Indeed, we observed a concentration-dependent decrease of TF expression in HAECs (Fig. 5A). However, contrary to ticagrelor, adenosine decreased TF RNA expression suggesting a transcriptional regulation of TF (Fig. 5 B). Further, we did not observe similar effects using the ENT1 inhibitor dipyridamole (Fig. 5C). Correspondingly, preincubation of HAECs using adenosine receptor antagonists against A_1 (8-cyclopentyl-1,3-dipropylxanthine, 10^{-7} M), A_{2A} (SCH 442416, 10^{-7} M), A_{2b} (MRS 1754, 10^{-7} M) and A_3 (VUF 5574, 10^{-7} M)[29,30] receptors individually (data not shown) or in combination did not reverse the effects of ticagrelor (Fig. 5D).

Ticagrelor decreases arterial thrombosis and endothelial TF expression in mice

Whole blood aggregometry after 2 weeks of treatment with ticagrelor 0.15% and clopidogrel 0.06% revealed equal and close to full (>97%) inhibition of ADP-induced platelet aggregation, as compared with animals treated with control chow (Fig. 6A); lower concentration, i.e. ticagrelor 0.1% and clopidogrel 0.04% provided subtotal platelet inhibitory effects (Fig. 6A). Treatment of rodents using ticagrelor 0.15% resulted in plasma concentration of 2.7 ± 0.7 uM (Fig. 6B). Also, platelet aggregation in response to thrombin (1U/mL) and collagen (10µg/mL) did not differ between ticagrelor- and clopidogrel-treated mice (data not shown). Mice treated with ticagrelor or clopidogrel showed prolonged arterial occlusion times, compared with animals treated with control chow. Moreover, despite equal inhibition of platelet aggregation, ticagrelor prolonged time to arterial occlusion, compared with clopidogrel-treated animals (Fig. 6C). In line with our *in vitro* finding, animals treated with ticagrelor, but not clopidogrel, revealed decreased endothelial TF expression in murine CCAs, as compared with rodents treated with control chow (Fig. 6D). Instead, plasma TF activity (Fig. 6E) and plasma thrombin generation (Fig. 6F) were unchanged between the groups suggesting similar plasma coagulation properties.

Discussion

Different $P2Y_{12}$ receptor antagonists have been shown to differentially affect cardiovascular outcome in several clinical trials[3,4]. Particularly, the Study of Platelet Inhibition and Patient Outcomes (PLATO) trial showed superior effects of ticagrelor, compared with clopidogrel, in reducing mortality in patients with ACS[3]. Stronger antiplatelet effects and reversible binding, which further allows inhibition of newly formed platelets and micro particles, may partly account for the greater efficacy of ticagrelor. Nonetheless, increased platelet inhibition achieved by prasugrel, compared with clopidogrel, did not translate into a similar reduction of overall mortality suggesting the possible involvement of platelet-independent effects[4]. In line with this, we hypothesized that ticagrelor may prevent endothelial activation with a specific effect on arterial thrombus formation and its key player TF; this indeed could provide a plausible explanation for the reduction in thrombotic events observed in PLATO.

Indeed, in TNF-α-stimulated HAECs, we found that ticagrelor, but not CAM, reduced TF expression via proteasomal degradation and TF activity without affecting TFPI. Interestingly, we found that these effects were mediated independently of both the $P2Y_{12}$ receptor and the adenosine transporter ENT1. To test the physiological relevance of our *in vitro* findings, we induced arterial thrombosis in C57BL/6 mice treated with ticagrelor or clopidogrel. Dosages were chosen to mimic human plasma concentrations[21] and to induce comparable platelet inhibition in both groups and, thus, to exclude platelet-dependent effects. Notably, we found that time to arterial occlusion was significantly delayed in ticagrelor-treated mice, compared with clopidogrel-treated rodents. In line with our *in vitro* findings ticagrelor, but not clopidogrel, reduced protein expression of endothelial TF in murine arteries. Plasma coagulation parameters including thrombin generation and tissue factor activity on the other hand remained unchanged, which further supports the hypothesis of local vascular antithrombotic mechanisms.

The herein reported findings support our hypothesis of pleiotropic effects of ticagrelor on endothelial cells and expand previous studies describing $P2Y_{12}$-independent effects of ticagrelor[7,8]. The inhibitory effect on TF displayed by ticagrelor may be particularly favourable in patients at risk of cardiovascular complications, as both activation of platelets and activation of the coagulation cascade represent key mechanisms in the initiation and propagation of arterial thrombus formation as it occurs in ACS. This notion, may contribute to the observed decrease in arterial thrombotic events[3].

Unlike previous observations[8,21], the herein reported mechanisms cannot be explained by increased adenosine levels following ENT1 inhibition. Although adenosine mimics the effect of ticagrelor, we did not observe similar results using the ENT1 inhibitor dipyridamole. Additionally, adenosine receptor antagonists could not reverse ticagrelor's effect; thus, implying a different mechanism. Correspondingly, patients receiving ticagrelor or adenosine show similar side effects, such as the induction of dyspnea[3], and the recent finding of increased adenosine levels[8] through ENT1 inhibition[7], provides evidence to link these observations. Nevertheless, potent ENT1 inhibitors such as dipyridamole also increase adenosine levels[8], but do not induce dyspnea[31]. Therefore, exclusive ENT1 in addition to $P2Y_{12}$ inhibition may not explain the entire mode of action of ticagrelor and further research is needed to determine its exact mechanisms.

Because of the chemical similarity of ticagrelor and adenosine, direct interactions with adenosine receptors appear to be likely and have been investigated by others; however, such interactions were considered unlikely to be clinically relevant because of the low affinity of ticagrelor[7]. The $P2Y_{12}$ receptor belongs to the family of P2 purine and pyrimidine G protein-coupled receptors and 7 further P2Y receptors have been characterized, some of which are expressed in the endothelium[32]. However, whether ticagrelor shows binding affinity to other purine and pyrimidine receptors and whether these receptors are responsible for the observed effects on endothelial cells remains to be determined.

Here we for the first time provide evidence for endothelium-dependent antithrombotic properties, which indicates that ticagrelor can affect the coagulation system in addition to its well-known antiplatelet effects. Dual antiplatelet therapy is the standard of care in patients suffering from ACS[2]; however, given the high prevalence of this disease[1], there is also a great number of patients with comorbidities requiring anticoagulant treatment, such as deep vein thrombosis, atrial fibrillation, or mechanical heart valves[33]. Although anticoagulants in combination with dual antiplatelet therapy lower the risk of thrombotic events, disproportionately increased bleeding rates have been observed[34]. While single antiplatelet therapy using clopidogrel in combination with anticoagulants reduced the risk of bleedings[35], such an approach may not be sufficient to sustain the low rate of thrombotic events. Thus, further studies are needed to determine the effectiveness and safety of new oral anticoagulants in combination with more potent platelet antagonists such as ticagrelor[33]. Because of its dual antithrombotic effects, ticagrelor may be a more appropriate choice than clopidogrel in patients with ACS with comorbidities requiring additional antithrombotic therapy. Along these lines, ticagrelor showed better efficacy than clopidogrel in the PLATO trial[3]. On the other hand, safety in terms of bleeding may be of particular concern in these patients as ticagrelor increased non-procedure-related bleeding rates in the PLATO trial[3].

In conclusion, the pleiotropic effects of ticagrelor on the endothelium may in part explain its greater efficacy in reducing thrombotic events in patients with ACS and mortality in clinical trials; its antithrombotic properties may be particularly promising in patients requiring anticoagulant in addition to antiplatelet therapy. Nonetheless, additional antithrombotic properties may increase bleeding risk and special attention may be required in patients at high risk.

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Conflict of interest

Ticagrelor was provided by AstraZeneca, Mölndal, Sweden and clopidogrel active metabolite by Sanofi-Aventis, Germany GmbH. TFL and JHB have received educational and research grants as well as honoraria from AstraZeneca, Zug, Switzerland.

Abbreviations

ACS	acute coronary syndrome
ACTB	human β-actin
ADP	adenosine diphosphate
CAM	clopidogrel active metabolite
CCA	common carotid artery
C_T	cycle threshold
ENT1	equilibrative nucleoside transporter 1
ETP	endogenous thrombin potential
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HAECs	human aortic endothelial cells
HBMVECs	human brain microvascular endothelial cells
HCMVECs	human cardiac microvascular endothelial cells
MFI	mean fluorescence intensity
PLATO	study of platelet inhibition and patient outcomes
RT-PCR	real-time polymerase chain reaction
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TNF-α	tumor necrosis factor-alpha

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Legends

Figure 1. Effects of ticagrelor and CAM on TF expression and activity in HAECs. TF protein expression in ticagrelor-pretreated HAECs (n=8) (A), or CAM-pretreated HAECs (n=8) (B) 1 hour before stimulation with TNF-α for 5 hours. (C) TF activity in ticagrelor-pretreated HAECs 1 hour before TNF-α stimulation for 5 hours. (n=8). TF protein expression in HAECs pretreated with the PI3 kinase inhibitor wortmannin (n=6) (D), the p70s6 kinase inhibitor rapamycin (n=6) (E) or both (n=8) (F) 1 hour prior to ticagrelor treatment for 1 hour and subsequent TNF-α stimulation for 5 hours. *p <0.05 vs TNF-α treatment; †p <0.05 vs TNF-α + ticagrelor treatment. CAM = clopidogrel active metabolite; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HAECs = human aortic endothelial cells; TF = tissue factor; TNF-α = tumor necrosis factor-alpha.

Figure 2. Ticagrelor augmented TF transcription but did not alter mRNA half-life in HAECs. (A) TF mRNA levels in HAECS after preincubation with ticagrelor 1 hour before TNF-α-stimulation for 3 hours (n=7). (B) TF mRNA stability after treatment with TNF-α (10 ng/mL) for 1 hour and subsequent simultaneous incubation with actinomycin D (10 μg/mL) and ticagrelor (n=4-6) for time points between 0 and 180 minutes. *p <0.05 vs TNF-α treatment. Act = actinomycin D; AU = arbitrary unit; HAECs = human aortic endothelial cells; TF = tissue factor; Tica = ticagrelor; TNF-α = tumor necrosis factor-alpha.

Figure 3. Ticagrelor reduces TF by proteasomal degradation in HAECs. (A) TF expression in HAECs stimulated with TNF-α for 3 hours followed by simultaneous treatment with the protein translation inhibitor cycloheximide and ticagrelor for additional 2 hours (n=9). (B) TF protein expression in HAECs stimulated with TNF-α-for 3 hours and treated with the proteasome inhibitor MG-132 and ticagrelor simultaneously for additional 2 hours (n=4). *p <0.05 vs TNF-α treatment. †p <0.05 vs. TNF-α + cycloheximide treatment. GAPDH =

glyceraldehyde 3-phosphate dehydrogenase; TF = tissue factor; TNF-α = tumor necrosis factor-alpha.

Figure 4. Presence of $P2Y_{12}$ receptor in human platelets but not in endothelial cells. (A) $P2Y_{12}$ RNA in human platelets compared with HAECs, HBMVECs and HCMVECs (n=3). (B) Protein expression of the $P2Y_{12}$ receptor in human platelets, compared with HAECs, HBMVECs or HCMVECs (n=3). ACTB = human β-actin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HAECs = human aortic endothelial cells; HBMVECs = human brain microvascular endothelial cells; HCMVECs = human cardiac microvascular endothelial cells.

Figure 5. Effects of adenosine, dipyridamole and adenosine receptor antagonists on TF expression. (A) Effects of adenosine on TF protein (n=10) and (B) RNA expression (n=4) after pretreatment for 1 hour and subsequent stimulation with TNF-α for 5 and 3 hours, respectively. (C) Effects of the ENT1 inhibitor dipyridamole on TF expression in HAECs after pretreatment for 1 hour and subsequent stimulation with TNF-α for 5 hours (n=6). (D) Pretreatment of HAECs with adenosine receptor antagonists for 1 hour prior to ticagrelor treatment for 1 additional hour and subsequent TNF-α stimulation for 5 hours (n=4). *p <0.05 vs TNF-α treatment. ACTB = human β-actin; ENT1 = equilibrative nucleoside transporter 1; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HAECs = human aortic endothelial cells; RA = receptor antagonists; TF = tissue factor; TNF-α = tumor necrosis factor-alpha.

Figure 6. Platelet aggregation, endothelial TF expression, time to arterial occlusion and thrombin potential. (A) Platelet aggregation in response to ADP (10 μM) in mice treated with control chow, clopidogrel or ticagrelor for 2 weeks. (B) Plasma concentration of ticagrelor in mice treated with ticaglrelor 0.15% added to chow. (C) Time to arterial thrombosis in mice treated with control chow (n=10), clopidogrel (n=7) or ticagrelor (n=8). (D)

Endothelial TF expression in CCAs of mice treated with control chow, clopidogrel or ticagrelor (n=5) and representative transverse sections of CCA stained for endothelium (CD31, red, scale bar 5 μm) and TF (green, scale bar 5 μm) (E). Plasma TF activity and (F) plasma thrombin potential clopidogrel-, ticagrelor- and control chow-treated animals at baseline. *p <0.05 vs control; †p <0.05 vs clopidogrel. ADP = adenosine diphosphate; CCA = common carotid artery; ETP = endogenous thrombin potential; MFI = mean fluorescence intensity; TF = tissue factor.

Figures

Figure 1

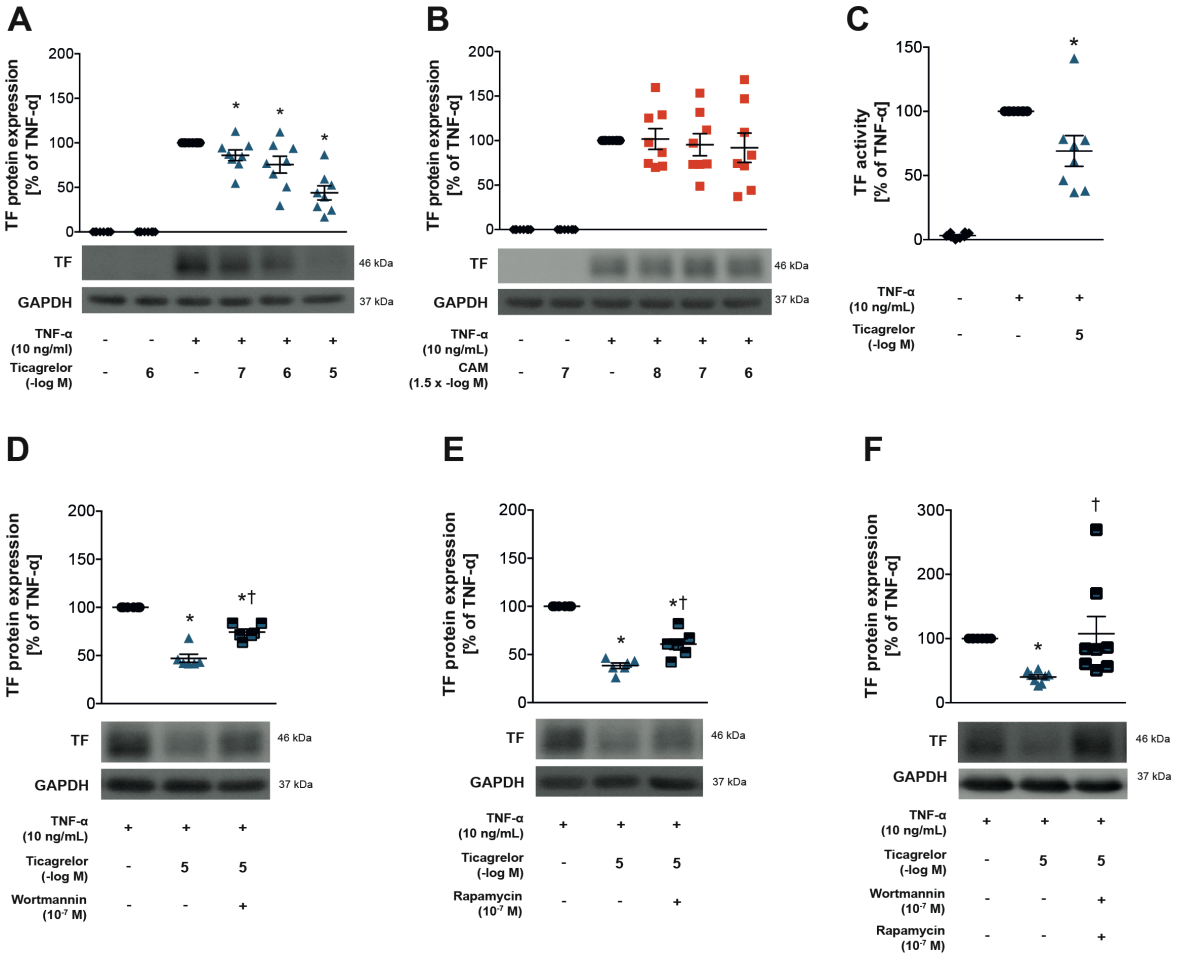


Figure 2

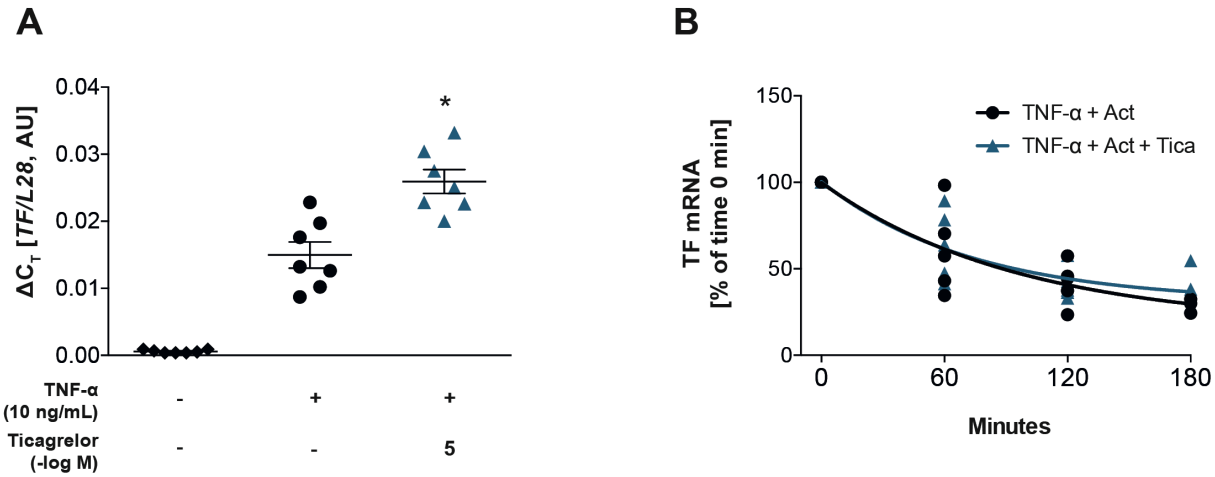


Figure 3

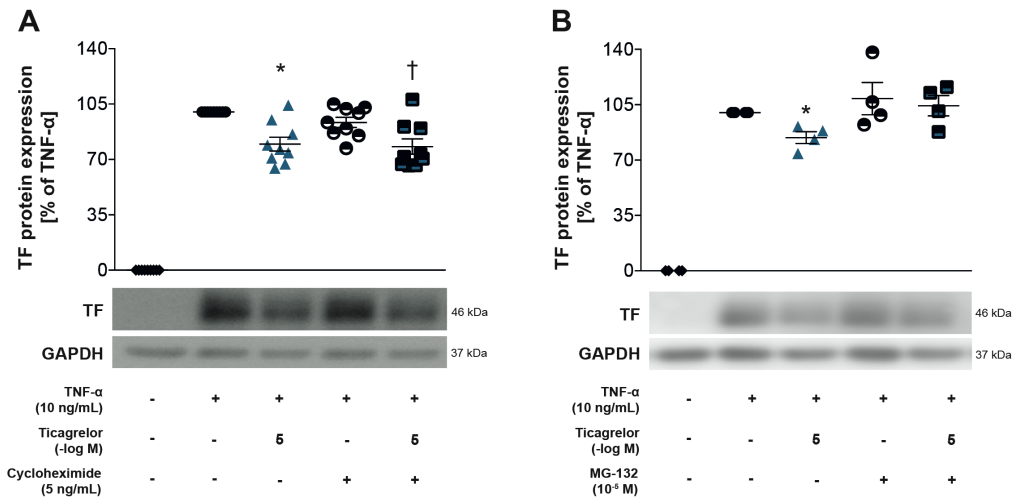


Figure 4

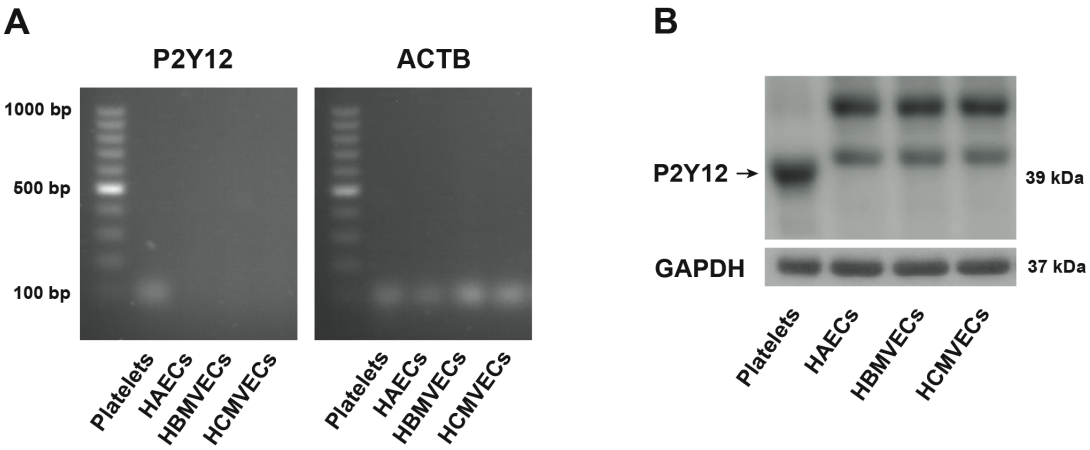
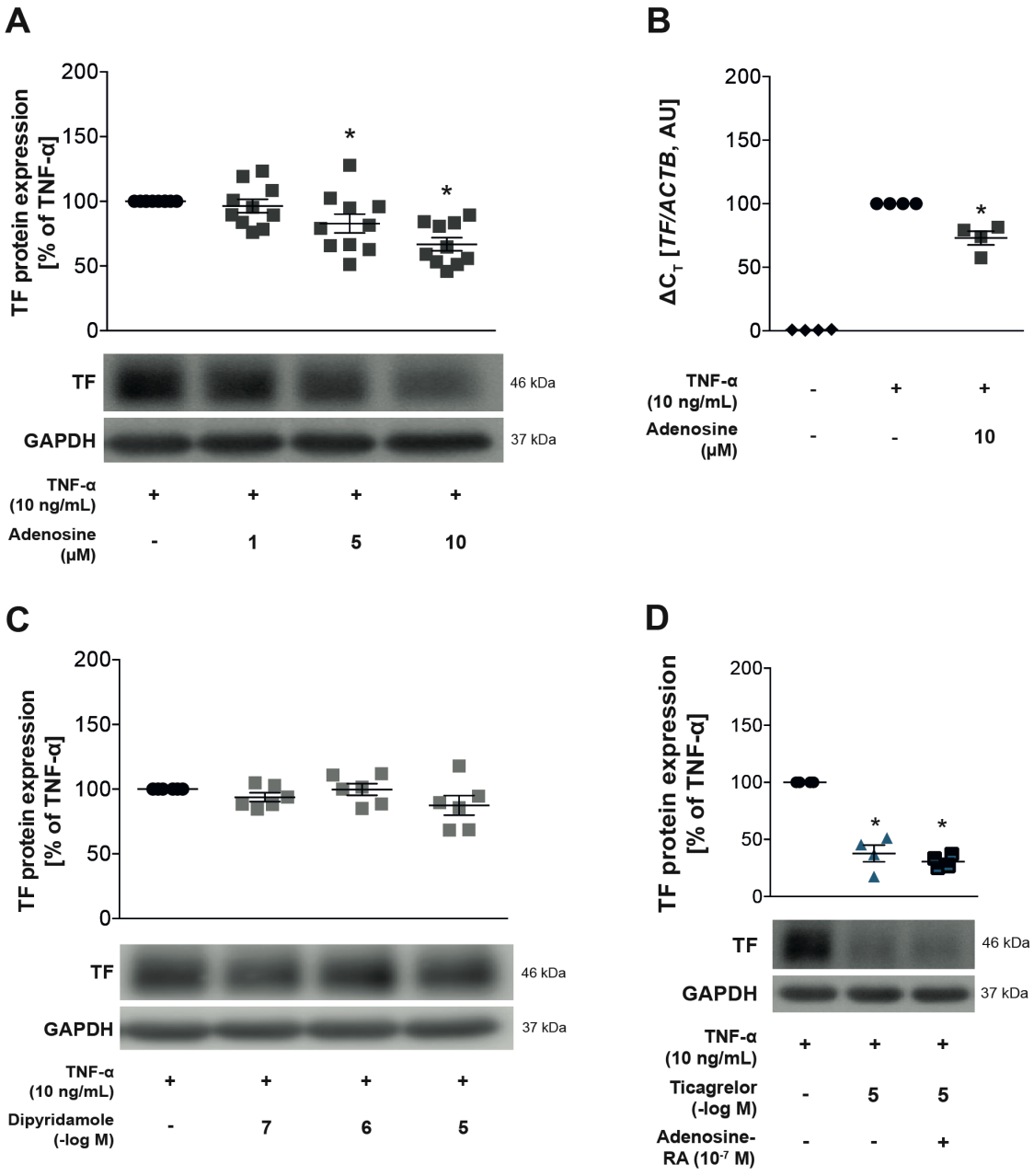


Figure 5


A
TF protein expression [% of TNF-α]
200
150
100
50
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46 kDa
GAPDH
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TF protein expression [% of TNF-α]
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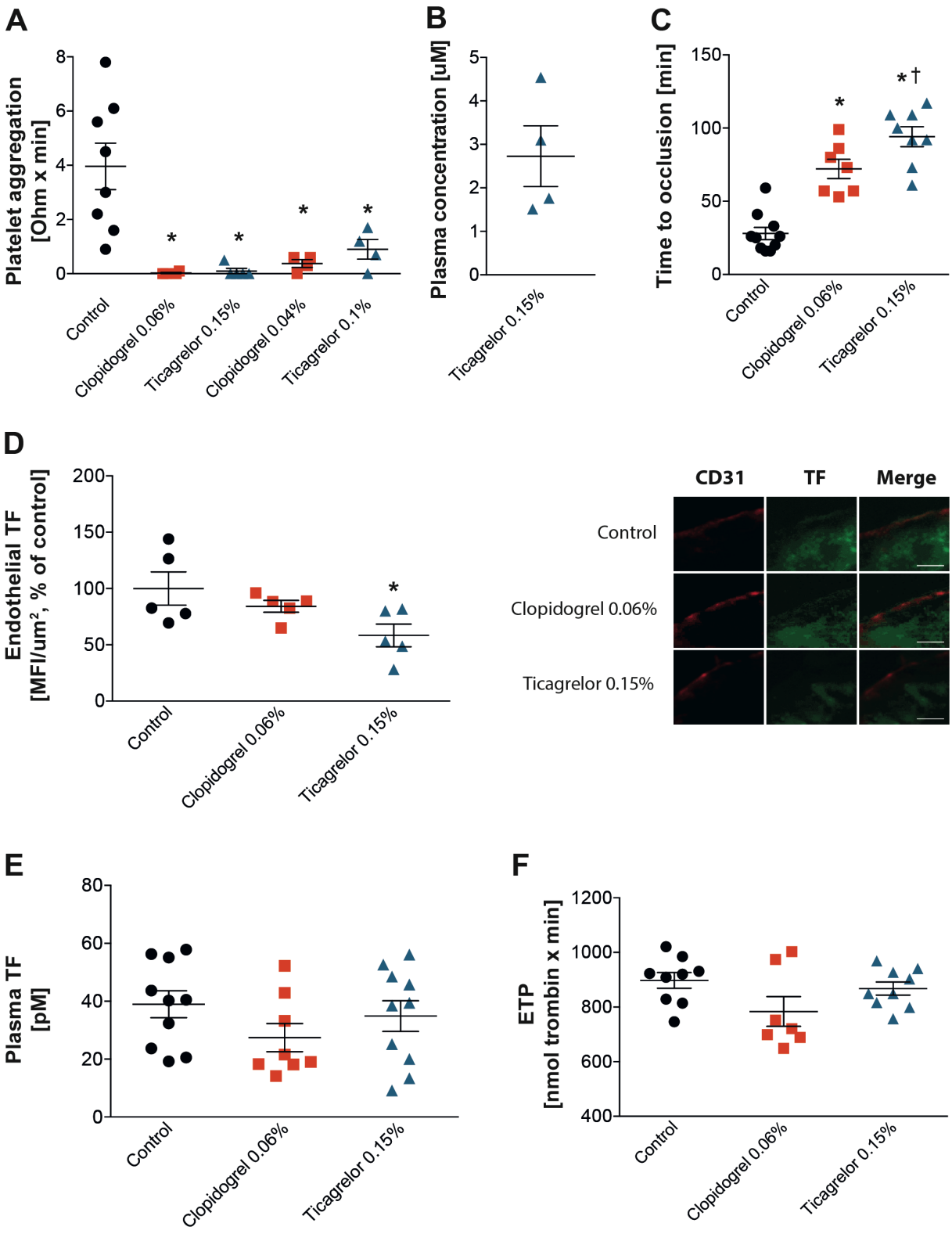
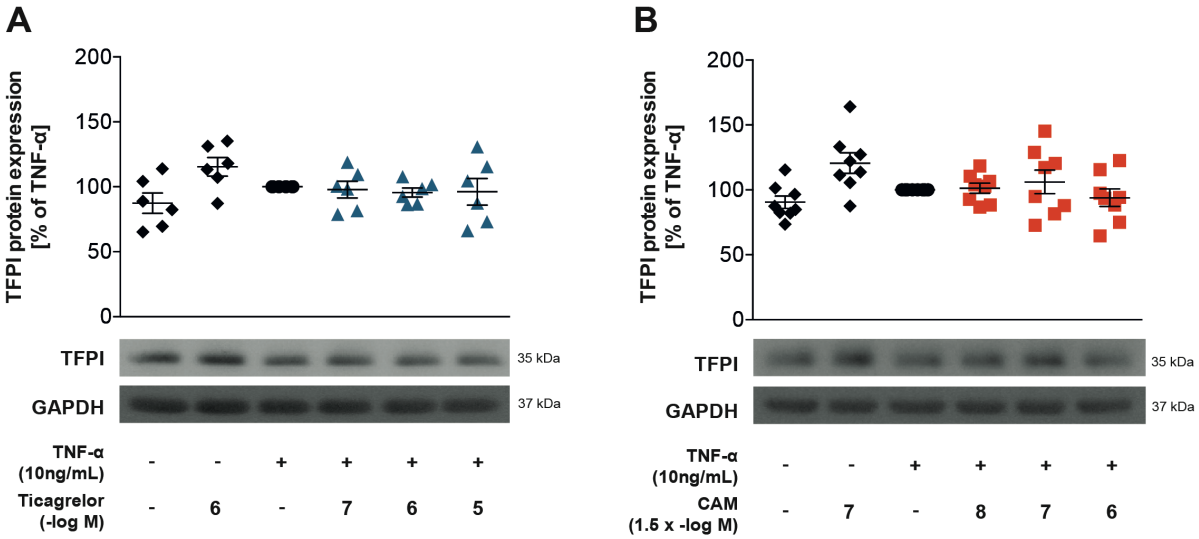
TNF-α (10 ng/mL)
+ + + +
Dipyridamole (-log M)
- 7 6 5
D
TF protein expression [% of TNF-α]
200
150
100
50
0
*
*
TF
46 kDa
GAPDH
37 kDa
TNF-α (10 ng/mL)
+ + +
Ticagrelor (-log M)
- 5 5
Adenosine-RA (10^-7 M)
- - +

Figure 6


A
Platelet aggregation [Ohm x min]
Control
Clopidogrel 0.06%
Ticagrelor 0.15%
Clopidogrel 0.04%
Ticagrelor 0.1%
B
Plasma concentration [uM]
Ticagrelor 0.15%
C
Time to occlusion [min]
Control
Clopidogrel 0.06%
Ticagrelor 0.15%
D
Endothelial TF [MFI/um², % of control]
Control
Clopidogrel 0.06%
Ticagrelor 0.15%
CD31
TF
Merge
Control
Clopidogrel 0.06%
Ticagrelor 0.15%
E
Plasma TF [pM]
Control
Clopidogrel 0.06%
Ticagrelor 0.15%
F
ETP [nmol trombin x min]
Control
Clopidogrel 0.06%
Ticagrelor 0.15%

Supplementary files

Supplementary figure 1



Supplementary figure 1. Effects of ticagrelor and CAM on TFPI expression in HAECs. TFPI expression in TNF-α-stimulated HAECs treated with ticagrelor (n=8) (A) or CAM (n=8) (B). CAM = clopidogrel active metabolite; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; HAECs = human aortic endothelial cells; TFPI = tissue factor pathway inhibitor; TNF-α = tumor necrosis factor-alpha.

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Ticagrelor, but not Clopidogrel Active Metabolite, Displays Antithrombotic Properties in the Left Atrial Endocardium

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Abstract

Aims

Oral anticoagulation is considered standard therapy for stroke prevention in atrial fibrillation (AF). Endocardial activation triggers expression of pro-thrombotic mediators including tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1), and contributes to thrombus formation in the left atrial appendage (LAA) of AF patients. Recently, pleiotropic effects of specific $P2Y_{12}$ receptor antagonists were demonstrated however, whether these drugs possess antithrombotic effects on LAA endocardial cells currently remains unknown.

Methods and results

LAA were obtained from 14 patients with known AF undergoing elective cardiac surgery including LAA removal at the University Hospital Zurich. LAA endocardial cells were isolated and pre-incubated with ticagrelor (10^{-7}, 10^{-6}, 10^{-5}M) or clopidogrel active metabolite (CAM) ($1.5x10^{-8}$, $1.5x10^{-7}$, $1.5x10^{-6}$M) before stimulation with tumour necrosis factor-alpha (TNF-α) (10 ng/mL). Finally, TF and PAI-1 expression and activity were analysed. Ticagrelor, unlike CAM, concentration-dependently decreased TNF-α-induced TF expression and TF activity in LAA endocardial cells. Further, ticagrelor, but not CAM reduced PAI-1 expression and enzyme activity in TNF-α-stimulated LAA endocardial cells. In contrast, TF pathway inhibitor (TFPI) remained unaffected by both dugs.

Conclusion

Ticagrelor, but not CAM, reduces expression and activity of TF and PAI-1 in LAA endocardial cells isolated from patients with AF, indicating possible local antithrombotic effects. Such pleiotropic properties of ticagrelor may contribute to a reduction in thromboembolic complications in patients with AF.

Abbreviations

AF	atrial fibrillation
CAM	clopidogrel active metabolite
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
LAA	left atrial appendage
PAI-1	plasminogen activator inhibitor-1
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TNF-α	tumour necrosis factor-alpha

Introduction

Atrial fibrillation (AF) is a common cardiac arrhythmia affecting approximately 2% of the general population in developed countries[1]. It increases the risk of thromboembolic complications such as cardiogenic stroke as well as heart failure and all-cause mortality[2]. Oral anticoagulation is superior to dual or single antiplatelet therapy[3] and is considered standard therapy to reduce thromboembolic complications and mortality in AF patients[2].

AF leads to thrombus formation in the left atrial appendage (LAA) due to a reduction in blood flow, endocardial activation and hypercoagulability mediated through cytokines such as tumour necrosis factor-alpha (TNF-α)[4]. We previously demonstrated that TNF-α-activated human LAA endocardial cells increase their levels of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) expression and activity, which may contribute to a higher thrombogenicity in left vs. right atrial appendages[5]. An increasing body of evidence is suggesting pleotropic effects of certain $P2Y_{12}$ receptor antagonists[6,7]; indeed, ticagrelor was shown to inhibit the adenosine transporter equilibrative nucleoside transporter 1[6,7] leading to increased plasma levels of adenosine[8]. In the current study, we investigated whether ticagrelor or clopidogrel active metabolite (CAM) affect the expression of prothrombotic mediators in TNF-α-activated human LAA endocardial cells.

Results

Ticagrelor, but not CAM, reduces TF expression and activity

Ticagrelor concentration-dependently decreased TNF-α-induced TF expression in LAA endocardial cells (Fig. 1A). The maximum effect amounted to 40% reduction in TF expression as compared to TNF-α stimulated cells. In contrast, CAM did not affect TF expression (Fig. 1B). In line with protein expression, ticagrelor, but not CAM, reduced TF activity (Fig. 1C). In contrast, endocardial protein expression of TFPI, the physiological antagonist of TF, was affected neither by ticagrelor nor CAM (Supplementary figure 1).

Ticagrelor, unlike CAM, decreases PAI-1 expression and activity

Similar to TF, ticagrelor also reduced PAI-1 expression in TNF-α-stimulated LAA endocardial cells (Fig. 1D). The maximum effect observed was a 67% reduction in PAI-1 expression as compared to TNF-α stimulated cells. In contrast, no effect on PAI-1 expression was observed with CAM (Fig. 1E). Similarly, ticagrelor, but not CAM reduced enzymatic PAI-1 activity in LAA endocardial cells (Fig. 1F).

Discussion

In this study we demonstrate for the first time that ticagrelor, but not CAM, reduces TNF-α-induced TF and PAI-1 expression and activity in LAA endocardial cells isolated from patients with AF, hinting towards a possible local antithrombotic effect of ticagrelor at the cellular level.

Current treatment strategies for stroke prevention in patients with AF focus on inhibition of clot formation through interference with the coagulation cascade using vitamin K antagonists or non-vitamin K oral anticoagulants[2,9,10]. Antiplatelet therapy, even dual anti-platelet therapy using aspirin and clopidogrel has been shown to be inferior to oral anticoagulation in stroke prevention in AF patients[3,11]. Newer generation antiplatelet agents such as ticagrelor, however, have not been evaluated for this indication.

Our current findings unravel a putative additional property of ticagrelor via which thromboembolic complications may be reduced, i.e. by decreasing local thrombogenicity of LAA endocardial cells through the reduction of crucial prothrombotic mediators such as TF and PAI-1. An increasing body of evidence suggests pleiotropic effects of ticagrelor[6,7]; indeed it was shown to inhibit the adenosine transporter equilibrative nucleoside transporter 1[6,7] thereby leading to increased adenosine plasma concentrations in cardiac patients[8]. The current results indicate the possibility of a local antithrombotic effect of ticagrelor in the LAA of AF patients. It currently remains unknown whether other anticoagulant drugs may have similar properties. A recent study reported that rivaroxaban, in contrast to dabigatran, reduced TF expression in endothelial cells[12]. However, these experiments were performed in human vein endothelial cells (instead of left atrial endocardial cells in our setting) and only reported changes in TF gene expression leaving out protein expression as well as enzyme activity[12]. Yet, these findings in congregate raise the possibility that local antithrombotic effects may be operative in the reduction of stroke risk observed with anticoagulants.

Twenty to 30% of patients with AF suffer from comorbidities such as coronary artery disease requiring antiplatelet therapy[2,9]. Optimal antithrombotic regimen in these patients is currently a matter of debate due to the high bleeding risk associated with combined anticoagulant and antiplatelet therapy[9]. While the role of new $P2Y_{12}$ antagonists such as ticagrelor has not yet been addressed[9] and is subject of ongoing studies[13], local antithrombotic mechanisms by ticagrelor in the LAA may be of particular interest (and value) in this specific patient population. Further studies are required to investigate whether the observed *ex vivo* effects of ticagrelor translate into improved patient outcome, including both thromboembolic as well as bleeding complications.

In summary, our results indicate that ticagrelor reduces the expression and activity of local procoagulant proteins in LAA endocardial cells, which may contribute to a reduction of thromboembolic complications in patients with AF. Our findings may instigate further research investigating the clinical implications of these findings, particularly for patients with AF in need of concomitant antiplatelet therapy.

Funding Sources

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Disclosures

Ticagrelor and CAM were provided by AstraZeneca, Mölndal, Sweden and Sanofi-Aventis, Germany, respectively. The study was supported by a research grant from AstraZeneca, Mölndal, Sweden. A.B. has received educational fees from Biosense Webster, Biotronik and Actelion. J.H.B and T.F.L. have obtained educational or research grants by companies involved in antithrombotic drugs or devices such as AstraZeneca, Bayer HealthCare, FRG, Biosense Webster, Boehringer-Ingelheim, Daiichi-Sankyo, Eli Lilly, and Pfizer. J.S. has received consultant and/or speaker fees from Amgen, AstraZeneca, Bayer HealthCare, Biotronik, Biosense Webster, Boehringer-Ingelheim, Boston Scientific, Bristol-Myers Squibb, Daiichi-Sankyo, Cook Medical, Medtronic, Novartis, Pfizer, Roche, Sanofi- Aventis, Sorin and St. Jude Medical, and is co-director of CorXL, and reports grant support through his institution from Bayer Healthcare, Biosense Webster, Biotronik, Boston Scientific, Daiichi Sankyo, Medtronic, and St. Jude Medical. GGC has received consultant fees from Cardiorentis AG.

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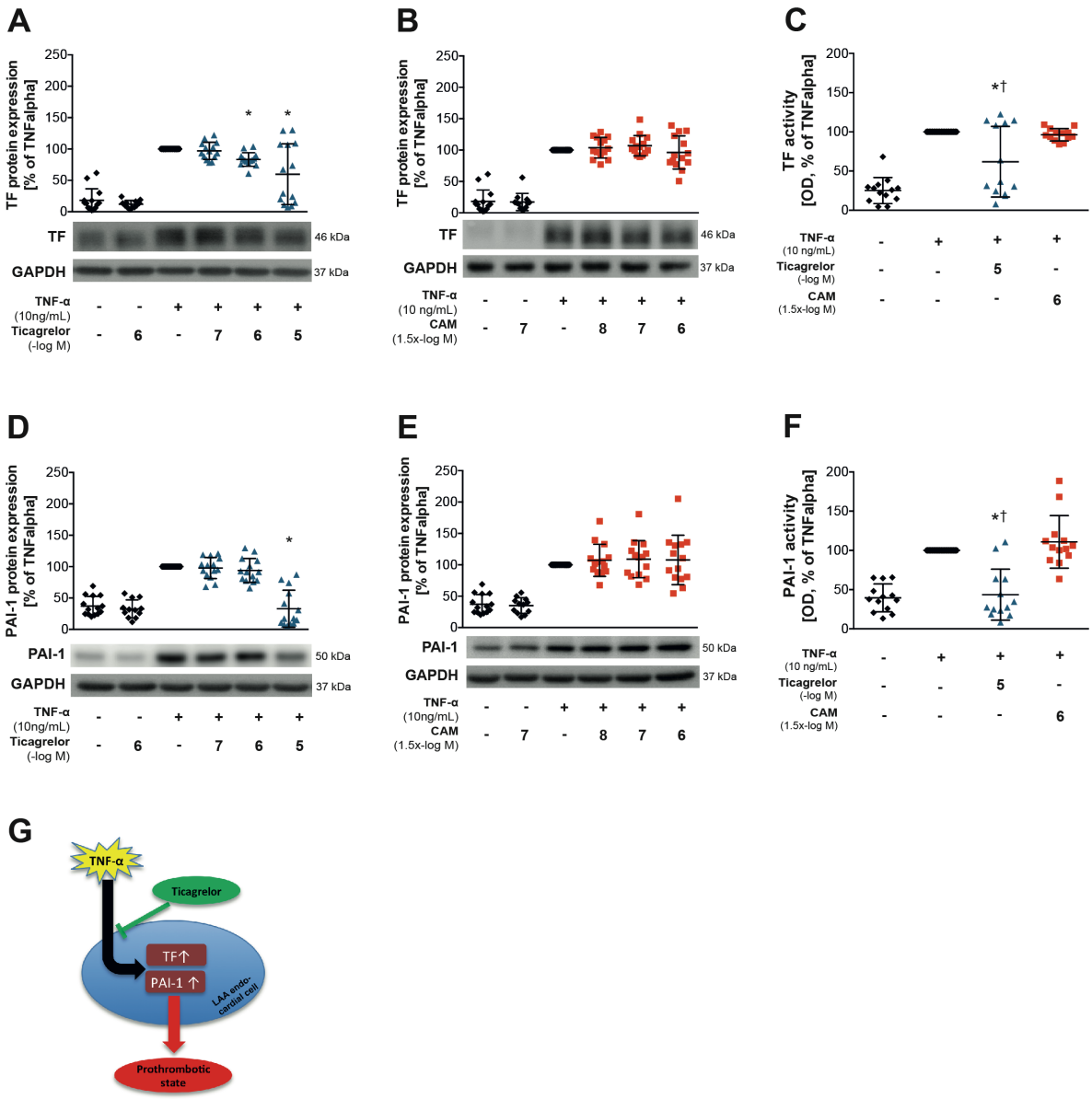
Figure and Figure Legend

Figure 1. Effects of ticagrelor and CAM on expression and activity of thrombotic mediators in LAA endocardial cells. (A) Western blotting analysis for TF protein expression in LAA endocardial cells pretreated with increasing concentrations of ticagrelor (n=14), or (B) CAM (n=14) with/without TNF-α. (C) ELISA for TF activity in LAA endocardial cells pretreated with ticagrelor or CAM with/without TNF-α stimulation (n=13). (D) Western blotting analysis for PAI-1 protein expression in LAA endocardial cells pretreated with increasing concentrations of ticagrelor (n=14), or (E) CAM (n=14) with/without TNF-α. (F) ELISA for PAI-1 activity in LAA endocardial cells pretreated with ticagrelor or CAM with/without TNF-α stimulation (n=13). (G) Schematic representation of main study finding. Data are expressed as mean ± SD. One-way ANOVA with Tukey post hoc test or unpaired two-tailed Student's t-test was applied. *p <0.05 vs TNF-α; †p <0.05 vs TNF-α + CAM. CAM = clopidogrel active metabolite; ELISA = enzyme-linked immunosorbent assay; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; LAA = left atrial appendage; PAI-1 = plasminogen activator inhibitor-1; SD = standard deviation; TF = tissue factor; TFPI = tissue factor pathway inhibitor, TNF-α = tumour necrosis factor-alpha.


A
TF protein expression [% of TNFalpha]
250
200
150
100
50
0
*
*
TF
46 kDa
GAPDH
37 kDa
TNF-α (10ng/mL) - - + + + +
Ticagrelor (-log M) - 6 - 7 6 5
B
TF protein expression [% of TNFalpha]
TF
46 kDa
GAPDH
37 kDa
TNF-α (10 ng/mL) - - + + + +
CAM (1.5x-log M) - 7 - 8 7 6
C
TF activity [OD, % of TNFalpha]
200
150
100
50
0
*†
TNF-α (10 ng/mL) - + + +
Ticagrelor (-log M) - - 5 -
CAM (1.5x-log M) - - - 6
D
PAI-1 protein expression [% of TNFalpha]
250
200
150
100
50
0
*
PAI-1
50 kDa
GAPDH
37 kDa
TNF-α (10ng/mL) - - + + + +
Ticagrelor (-log M) - 6 - 7 6 5
E
PAI-1 protein expression [% of TNFalpha]
PAI-1
50 kDa
GAPDH
37 kDa
TNF-α (10ng/mL) - - + + + +
CAM (1.5x-log M) - 7 - 8 7 6
F
PAI-1 activity [OD, % of TNFalpha]
200
150
100
50
0
*†
TNF-α (10 ng/mL) - + + +
Ticagrelor (-log M) - - 5 -
CAM (1.5x-log M) - - - 6
G
TNF-α
Ticagrelor
TF↑
PAI-1 ↑
LAA endothelial cell
Prothrombotic state

Supplementary Material

Material and Methods

Drugs

Ticagrelor and CAM were provided by AstraZeneca, Mölndal, Sweden and Sanofi-Aventis, Germany, respectively.

Study participants

LAA were obtained from 14 patients with known AF undergoing elective cardiac surgery (coronary artery bypass grafting and/or valve surgery, or surgical AF ablation and LAA occlusion) including LAA removal at the University Hospital Zurich[1]. All participants signed an informed consent form and the study was approved by the cantonal ethical committee Zurich "Kantonale Ethikkommission Zürich".

Isolation of left atrial appendages

LAA endocardial cells were isolated as previously described[1]. The surgical procedure, including removal of the LAA, was performed according to standard operating procedures and to the discretion of the cardiac surgeon in charge. After amputation, appendages were stored in low glucose DMEM medium (Gibco®, Life Technologies, Switzerland) enriched with 10% fetal bovine serum.

Isolation of human atrial endocardial cells

Human LAA endocardial cells were isolated as previously described[1]. Briefly, after removal of DMEM medium, LAAs were washed with pre-warmed washing buffer (HBSS [Ca^{2+}- and Mg^{2+}-free, with phenol red] enriched with 10 mM HEPES and 0.1% BSA, pH 7.4); the lumen was placed in collagenase–dispase solution (0.4%) and incubated for 35 min at 37°C[1].

Following incubation, cells were collected, cell suspension was centrifuged (5min, 233rcf, 4°C) and resuspended in 2 mL of medium (DMEM low glucose, 20% fetal bovine serum, 100µg/mL heparin, 25mM HEPES, pH 7.4, 1 time non-essential amino acids and 1 time penicillin-streptomycin) enriched with 20 µL/mL endothelium cell growth factor (Sigma-Aldrich, Switzerland)[1]. Finally, cells were seeded on a gelatine-coated dish (0.1% gelatine, bovine skin type B [Sigma-Aldrich, Switzerland])[1]. The herein described isolation procedure yielded endocardial cells of 98% purity[1].

Cell culture experiments

LAA endocardial cells were used for experiments between passage 3 and 4. Cells were grown to 80% confluence on gelatine-coated six well plates (TPP, Trasadingen, Switzerland) and underwent starvation using growth medium containing 0.5% fetal bovine serum for 16 to 18 hours. Cells were pre-incubated with ticagrelor (10^{-7}, 10^{-6}, 10^{-5} M) or CAM ($1.5x10^{-8}$, $1.5x10^{-7}$, $1.5x10^{-6}$ M) 1 hour before stimulation with TNF-α (10 ng/mL) (R&D Systems, Minneapolis, MN) for 5 hours. Drug concentration ranges were chosen so as to match plasma concentrations found in humans[2]. Drugs were dissolved in dimethyl sulfoxide at concentrations (0.1%) known not to affect TF, TF pathway inhibitor (TFPI) or PAI-1 protein expression[3]. Correspondingly, unstimulated control cells and TNF-α-stimulated cells were treated with dimethyl sulfoxide (0.1%) to exclude any other vehicle-dependent effects.

Western blotting

Protein expression was determined by Western blot analysis as previously described[1]. Endocardial cells were incubated with lysis buffer (NaCl 150 mM, EDTA 1 mM, NaF 1 mM,DTT 1 mM, aprotinin 10 mg/mL, leupeptin 10 mg/mL, $Na_3VO_4$0.1 mM, PMSF 1 mM, and NP-40 0.5%); protein concentration was determined according to the manufacturer's recommendations (Bio-Rad); 20 – 30 μg of protein lysates were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferredto a

polyvinylidene fluoride membrane by semi-dry transfer. Membranes were incubated with primary antibodies overnight at 4°C on a shaker. Secondary antibodies were applied for 1 hour at room temperature. Densitometric analyses were performed and protein expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Antibodies against TF (ADG4503; 1:2000), TFPI (ADG72; 1:8000) were purchased from American Diagnostica; anti-PAI-1 (1:500) from Santa Cruz Biotechnology; anti-GAPDH antibody (MAB374; 1:40000) from Merck Millipore. Secondary anti-mouse (1031-05) and anti-rabbit (4050-05) antibodies were obtained from SouthernBiotech.

Tissue factor activity assay

TF activity was determined according to the manufacturer's recommendations (Sekisui Diagnostics, ACTICHROME® TF, 846). Endocardial cells were lysed (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1% Triton X-100, pH 7.4), diluted 1:15 in assay buffer, and mixed with human factor VIIa and X, which leads to conversion of factor X to Xa; factor Xa subsequently cleaves the chromogenic substrate SPECTROZYME® FXa. Finally, absorbance was measured at 405 nm and after background subtraction, optical density was normalized to protein concentration as determined according to the manufacturer's recommendations (Bio-Rad).

Plasminogen activator inhibitor-1 activity assay

PAI-1 activity was determined according to the manufacturer's recommendations (Sekisui Diagnostics, SPECTROLYSE® PAI-1 activity assay). Briefly, 30 µg of protein lysates were incubated with tissue plasminogen activator allowing reaction with PAI-1 in the samples. Residual tissue plasminogen activator activity was then measured after adding human glu-plasminogen, poly-D-lysine and a chromogenic substrate for plasmin. Residual tissue plasminogen activator catalysed the conversion of plasminogen to plasmin, which subsequently hydrolysed the chromogenic substrate. PAI-1 activity is thus inversely

proportional to residual tissue plasminogen activator activity. Absorbance was measured at 405 nm and following background subtraction, optical density was normalized to protein concentration as determined according to the manufacturer's recommendations (Bio-Rad).

Statistical analysis

Data are expressed as mean ± standard error of the mean. Statistical analysis was performed using one-way ANOVA with Tukey post hoc test or unpaired two-tailed Student's t-test as appropriate. A probability value ≤0.05 was considered as statistically significant and calculated by Prism 6 (GraphPad software).

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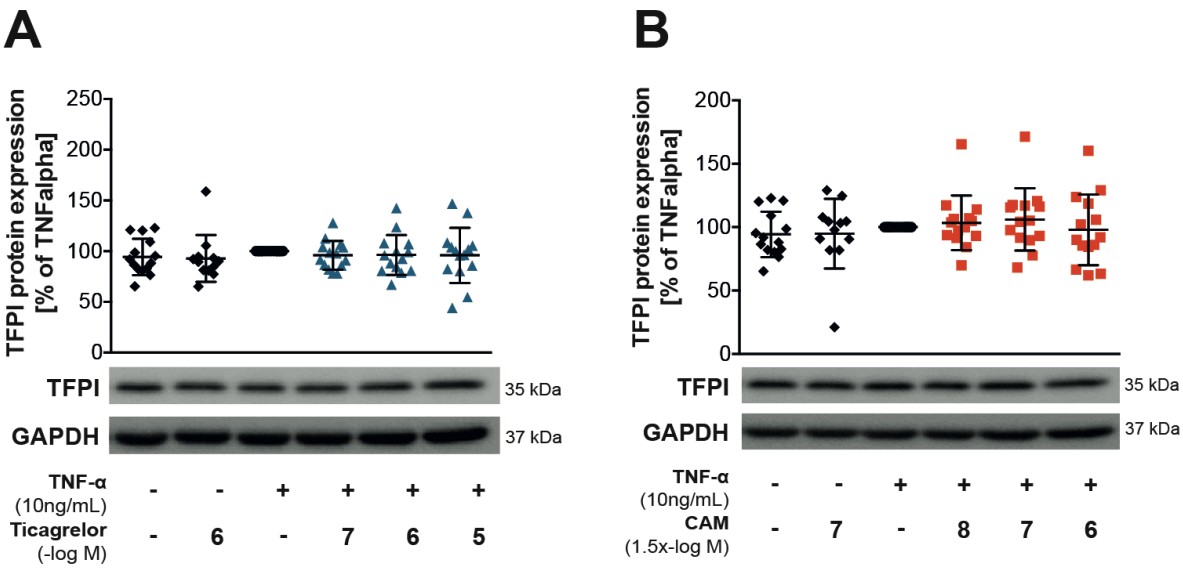
Supplementary Figure and Figure Legend

Supplementary Figure 1



Supplementary Figure 1. Effects of ticagrelor and CAM on TFPI protein expression in LAA endocardial cells. (A) Western blotting analysis for TFPI protein expression in LAA endocardial cells pretreated with increasing concentrations of ticagrelor (n=14), or (B) CAM (n=14) with/without TNF-α. Data are expressed as mean ± SD. Unpaired two-tailed Student's t-test was applied. *p <0.05 vs TNF-α. CAM = clopidogrel active metabolite; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; LAA = left atrial appendage; SD = standard deviation; TFPI = tissue factor pathway inhibitor, TNF-α = tumour necrosis factor-alpha.

6 Summary

6.1 Ticagrelor, but not clopidogrel active metabolite, reduces endothelial tissue factor via proteasomal degradation

In our initial study we showed that ticagrelor, unlike CAM, dose-dependently reduced TF protein expression as well as TF enzyme activity in TNF-α-stimulated HAECs indicating local antithrombotic properties of ticagrelor on the human endothelium[1] in addition to its well-described anti-platelet effects.[2] By investigating the underlying molecular mechanisms, we found that the reduction in endothelial TF expression by ticagrelor was reversed by wortmannin and rapamycin suggesting the involvement of the signalling molecules PI3K and p70s6 kinase.[1] Furthermore, inhibition of proteasomes using MG-132 reversed the effects of ticagrelor on TF expression in HAECs.[1] On the other hand, ticagrelor did not decrease TF mRNA nor did it affect TF mRNA stability indicating that ticagrelor reduced endothelial TF expression by proteasomal degradation rather than mRNA modification.[1] Interestingly, the observed effects of ticagrelor were mediated independently of the ADP receptor $P2Y_{12}$,[1] the target receptor of ticagrelor on platelets[3], since it was not expressed in HAECs, neither on the mRNA nor on the protein level.[1] Recently it was shown that ticagrelor also inhibits the adenosine transporter ENT1,[4] which mediates cellular uptake of adenosine in red blood cells, among others,[5] thereby increasing adenosine plasma levels in patients treated with ticagrelor.[6] These findings led to the hypothesis that ticagrelor may inhibit ENT1, known to be expressed in endothelial cells,[7] and subsequently increases extracellular adenosine levels, which in turn affect TF expression through one of the four endothelial adenosine receptors[8]. In line with previous reports,[9] adenosine dose-dependently decreased TF expression in TNF-α-stimulated HAECs.[1] However, adenosine, unlike ticagrelor, exerted its effect via a reduction of TF mRNA.[1] In contrast to ticagrelor, dipyridamole, a slightly more potent ENT1 inhibitor[4], did not reduce TF protein expression in HAECs and thus, not mimic the effects of ticagrelor.[1] Lastly, inhibition of all adenosine receptors individually or in combination did not reverse the

reduction of TF mediated by ticagrelor thereby disproving the ENT1 hypothesis in endothelial cells.[1]

6.2 Ticagrelor, compared with clopidogrel, decreases endothelial tissue factor expression and arterial thrombosis in mice

Next, we investigated the physiological relevance of our *in vitro* findings in a mouse model of photochemical-induced arterial thrombosis and found that ticagrelor, but not clopidogrel, at physiological plasma concentrations, reduced TF expression in the endothelium of common carotid arteries.[1] Furthermore, ticagrelor prolonged time to arterial occlusion, compared with clopidogrel, in mice.[1] Plasma TF activity and endogenous thrombin potential, on the other hand, were comparable among the treated groups.[1] Likewise, platelet aggregation in response to ADP was inhibited to similar extents in both mice treated with ticagrelor and clopidogrel.[1] These findings indicate that ticagrelor exerts more potent antithrombotic properties, compared with clopidogrel, which was associated with a local reduction of endothelial TF expression and independent of changes in plasma TF activity, systemic coagulation or inhibition of ADP-induced platelet aggregation.[1]

6.3 Ticagrelor, unlike clopidogrel active metabolite, reduces thrombogenicity in atrial fibrillation patients

AF triggers thrombus formation in LAA leading to systemic embolism such as ischemic stroke causing substantial morbidity and mortality.[10] Although anticoagulant treatment is considered standard therapy to prevent thromboembolic complications[10], patients with AF frequently have comorbidities such as ACS requiring antiplatelet therapy.[11] Therefore, we studied whether certain $P2Y_{12}$ receptor antagonist may possess local antithrombotic properties in endocardial cells isolated from LAA of AF patients.[12] Recently it was shown that the thrombotic factors TF and plasminogen activator inhibitor-1 (PAI-1) are expressed to a higher extent in left, as compared with right atrial appendages, which may in part explain the greater

thrombogenicity observed in LAA.[13] In accordance with our previous findings in HAECs and a mouse model of arterial thrombosis[1] we found that ticagrelor, unlike CAM, reduced TF and PAI-1 protein expressions as well as enzyme activities in TNF-α-stimulated LAA endocardial cells isolated from AF patients.[12] On the other hand we did not observe different expression levels of TFPI, the physiological antagonist of TF.[12] Indeed, this translational study indicates local antithrombotic properties of ticagrelor, which may lower thrombogenicity in AF patients.[12]

7 Discussion

7.1 Ticagrelor exhibits platelet-independent antithrombotic effects on the endothelium

Cardiovascular disease including coronary artery disease and stroke among others is the leading cause of death in both men and women in Europe.[14] Arterial thrombus formation following rupture of an atherosclerotic plaque is the key pathophysiological mechanisms leading to an ACS.[15] Platelets, in addition to the coagulation system, are crucially involved in thrombus formation and inhibition of platelet aggregation has been proven to reduce thrombogenesis and thus MI and stroke.[16-19] In 1988 the second international study of infarct survival (ISIS-2) study showed that platelet inhibition by aspirin reduces non-fatal MI, non-fatal stroke and vascular mortality, compared with placebo.[16] Later, dual antiplatelet therapy (DAPT) using the $P2Y_{12}$ receptor antagonist clopidogrel in addition to aspirin was reported to further reduce non-fatal MI, stroke and CV death in the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) study.[17] In more recent years, however, newer generations of $P2Y_{12}$ receptor antagonists have evolved and were compared with the thienopyridine clopidogrel.[18,19] Prasugrel is a third generation thienopyridine and like clopidogrel requires cytochrome P450 metabolism in order to form its active metabolite, which irreversibly binds the platelet ADP receptor $P2Y_{12}$.[2] Yet, prasugrel only requires a one-step activation, as compared with the two-step activation of clopidogrel[20] and platelet inhibition occurs faster and to a higher extend; also, inter-individual variability and non-responsiveness to prasugrel is lower.[21,22] Consequently, prasugrel reduced the incidence of death from cardiovascular causes, non-fatal MI, or non-fatal stroke in patients with ACS, as compared with clopidogrel.[19] Nevertheless, prasugrel also increased major bleeding rates and thus, overall mortality was comparable in prasugrel- and clopidogrel treated patients.[19] In contrast, ticagrelor is a cyclopentyl-triazolo-pyrimidine, which does not require cytochrome P450 activation and binds the $P2Y_{12}$ receptor directly and reversibly[2]. Like prasugrel, ticagrelor displays faster and greater inhibition of ADP-induced platelet aggregation[23] and

inhibits newly formed platelets more efficiently in patients with high platelet turnover[24]. Correspondingly, ticagrelor, compared with clopidogrel, further reduced the incidence of death from vascular causes, MI and stroke in ACS patients in the Platelet Inhibition and Patient Outcomes (PLATO) study.[18] Importantly, major bleedings were not higher and ticagrelor reduced overall mortality.[18] Despite the fact that both prasugrel and ticagrelor show greater platelet inhibition compared with clopidogrel,[2] only ticagrelor reduced overall mortality[18,22]. Therefore, we hypothesized that platelet-independent effects of ticagrelor may contribute to this observation.

Indeed, we found that ticagrelor reduced endothelial TF expression and activity by proteasomal degradation in HAECs and that ticagrelor decreased endothelial TF expression in murine arteries subsequently prolonging time to arterial occlusion in mice, compared with clopidogrel.[1] Furthermore, ticagrelor lowered TF and PAI-1 expression in endocardial cells isolated from LAA of AF patients.[12] These results strongly indicate local antithrombotic properties of ticagrelor on endothelial and endocardial cells and may in part explain the reduced mortality observed in PLATO.

7.2 Ticagrelor-mediated tissue factor reduction in endothelial cells and its underlying molecular mechanisms

We found that ticagrelor, but not CAM, concentration-dependently reduced TF expression as well as TF activity in HAECs and subsequently investigated the underlying molecular mechanisms.[1] We found that the signalling molecules PI3K and p70s6 were involved in the reduction of TF protein expression by ticagrelor.[1] This observation pointed towards an involvement of the $P2Y_{12}$ receptor, a metabotropic G-protein coupled receptor linked to the downstream signalling molecule PI3K in platelets[25]. Nevertheless, we did not detected $P2Y_{12}$ mRNA or protein in endothelial cells.[1]

Recently, platelet- as well as $P2Y_{12}$ receptor-independent pleiotropic effects have been described for ticagrelor.[26] Unlike thienopyridines, ticagrelor also binds to the adenosine

transporter ENT1,[4] which is expressed in red blood cells and endothelial cells among others.[5,7] ENT1 mediates adenosine uptake in red blood cells, which was decreased by ticagrelor *in vitro*[27] and correspondingly, higher adenosine plasma levels were found in ACS patients after treatment with ticagrelor.[6] Adenosine is known to inhibit platelet aggregation[28] and indeed, adenosine contributed to the inhibition platelet aggregation in whole blood treated with ticagrelor[29]. Since both ENT1 and adenosine receptors are expressed in endothelial cells[7,8], we tested the hypothesis that ticagrelor inhibits ENT1 in HAECS increasing extracellular levels of adenosine and subsequently reducing TF via endothelial adenosine receptors. Indeed, adenosine decreased endothelial TF expression; however; this observation was mediated via a reduction in TF mRNA rather than proteasomal degradation as observed with ticagrelor[1]. Also, the ENT1 inhibitor dipyridamole[4] did not mimic, and inhibition of adenosine receptors did not reverse the effects of ticagrelor on TF expression suggesting that our observations were occurring independently of ENT1.[1] Indeed, ticagrelor shows side effects similar to adenosine such as dyspnoea[18,30,31] and increased adenosine levels through inhibition of ENT1 by ticagrelor[4] represent a plausible explanation for this finding. Nevertheless, several arguments speak against this hypothesis. Firstly, the ENT1 inhibitor dipyridamole does not cause dyspnoea[32,33] and secondly, the adenosine triphosphate analogue cangrelor, which, like ticagrelor, inhibits $P2Y_{12}$ receptors reversibly but does not bind to ENT1[4] also causes dyspnoea, albeit to a lesser extent.[34,35] These observation further support the concept that ticagrelor may exert additional mechanism beyond $P2Y_{12}$ and ENT1 inhibition.

Other molecular mechanisms potentially explaining our *in vitro* findings include direct binding of ticagrelor to adenosine receptors due to their molecular similarity. Indeed, ticagrelor shows low binding affinity to the adenosine receptors A_1, A_{2a}, A_{2b} and A_3; however clinical concentrations of ticagrelor are of neglectable functional relevance.[4] Moreover, ticagrelor may exhibit binding affinity to other family members of the P2 purine and pyrimidine

receptors expressed in endothelial cells.[36] However, to date no such interactions have been described.

7.3 Ticagrelor and arterial thrombosis *in vivo* – relevance of endothelial tissue factor

In C57BL/6 mice we found that ticagrelor reduced arterial thrombosis more effectively than clopidogrel and, correspondingly, that ticgrelor-treated mice showed lower endothelial TF expression.[1] On the other hand, we did not observe differences in platelet aggregation or systemic coagulation including plasma TF activity and endogenous thrombin potential between the two groups.[1] These results suggest that ticagrelor exerts local antithrombotic properties by reducing endothelial TF expression in addition to its well-described antiplatelet effects.[1]

TF plays a crucial role in arterial thrombosis as it initiates the extrinsic coagulation cascade by activating factor VII[37] followed by factor IX and X[38] and subsequently thrombin; thrombin in turn, activates a positive feedback loop[39] finally leading to large amounts of fibrin formation[40] in addition to platelet activation.[41] TF is expressed in the entire vasculature including endothelial cells[42], VSMC and adventitial cells[43] as well as on circulating TF-containing microparticles released from endothelial cells[44]; In pathological conditions, such as high shear flow[45] as occurring in a stenosed artery and during inflammation as occurring in atherosclerosis, TF expression is upregulated.[46] Consequently, TF is detected in the necrotic core of atherosclerotic plaques.[43,47,48] Vascular, rather than blood cell-derived TF appears to play a relevant role in arterial thrombus formation.[49] Day and colleagues showed that mice expressing low amounts of TF in bone marrow cells did not have prolonged arterial occlusion times and that mice with TF deficiency that were transplanted with bone marrow cells expressing physiologic amounts of TF, did not have reduced arterial occlusion times.[49] Importantly, the group used the same experimental mouse model for arterial thrombosis as we did in our study, i.e. photochemical injury of the carotid artery by bengal rose.[49] The

relevance of TF in arterial thrombosis was further supported by Pawashe et al. showing that anti-TF antibody treatment decreased arterial thrombosis in common carotid arteries of rabbits[50]. Lastly, clinical studies found an association of TF with cardiovascular risk factors[51-54] and the incidence of MI.[55]

Whether indeed the decrease of endothelial TF expression by ticagrelor entirely explains the reduction in arterial thrombosis observed in mice remains to be proven by rescue experiments. Since the exact mechanisms leading to TF degradation are not entirely understood, it appears difficult to rescue endothelial TF reduction *in vivo* without affecting other sources of TF. On the other hand, one would expect no differences in arterial thrombus formation in endothelial-specific TF knockout mice treated with ticagrelor or clopidogrel and exposed to photochemical injury of the common carotid arteries. Yet, we did not have access to such a mouse strain.

Although we have ruled out other potential and relevant mechanisms explaining the differences in arterial thrombus formation between ticagrelor- and clopidogrel-treated rodents, such as alterations in platelet aggregation, plasma TF activity and systemic coagulation, additional possible explanations remain. Kirby et al. reported that inhibition of platelet aggregation by NO, which is usually rather low, increased significantly in the presence of the $P2Y_{12}$ receptor antagonist prasugrel active metabolite or ticagrelor.[56] This finding was explained by synergistic effects of NO and $P2Y_{12}$ receptor inhibition on the reduction of the downstream signalling molecule PI3K.[56] NO is produced in endothelial cells by endothelial NO synthase[57] and inducible NO synthase during inflammation[58]. Interestingly, we have previously shown that ticagrelor, unlike CAM, dose-dependently increases phosphorylation of endothelial NO synthase at the activation side serine 1177.[59] In line with our findings, treatment with ticagrelor augmented myocardial NO synthase in rats.[60] Increased phosphorylation of endothelial NO synthase may result in higher concentration of NO at the vessel wall and may contribute to higher platelet inhibition *in vivo.*

Drug dosages of P2Y12 receptor antagonists in rodents

In humans, ticagrelor, compared with clopidogrel, exerts greater inhibition of ADP-induced platelet aggregation[23]. Therefore, different effects on MI, stroke and CV death between clopidogrel- and ticagrelor-treated patients[18,30] may be due to different platelet inhibitory effects. To rule out platelet-dependent effects in our study we selected dosages of ticagrelor and clopidogrel that resulted in comparable inhibition of ADP-induced platelet aggregation.[1] Importantly, we performed platelet aggregometry in whole blood as it was recently reported that adenosine contributes to whole blood platelet inhibition in the presence of ticagrelor.[29] In order to show that our experiments were performed at minimal drug concentrations and that none of the drugs were used at excessively high concentrations, we performed dose response experiments and found that reducing drug dosages by only one third resulted in residual and comparable platelet reactivity in ticagrelor- and clopidogrel-treated animals.[1] Besides, dosages in our *in vitro* and *in vivo* experiments were chosen so as to reflect plasma concentration found in humans. Recommended dosages in cardiovascular patients for clopidogrel and ticagrelor represent a loading dosage of 300 – 600 mg followed by 75 mg once daily and a loading dosage of 180 mg followed by 90 mg twice daily, respectively.[11] Thus, maintenance dosages differ 2.4 fold. Such dosages provide plasma concentration of 0.16 – 0.18 uM of clopidogrel active metabolite and 1 – 1.5 uM of ticagrelor in humans[2]. Likewise, drug dosages in our study differed 2.5 fold (ticagrelor 0.15% vs. clopidogrel 0.06%).[1] However, unlike in humans,[23] these dosages resulted in equivalent platelet inhibition in mice[1], which was also in line with previous findings in rats.[60] In addition, we evaluated ticagrelor plasma concentration in mice and found concentrations of 2.7±0.7 uM,[1] which were comparable to humans.[2]

8 Outlook

Here we report that ticagrelor, unlike CAM, reduces endothelial TF by proteasomal degradation *in vitro*.[1] On a molecular level we found that the effect of ticagrelor on TF was mediated by the signalling pathways PI3K and p70s6 kinase.[1] We have shown that the observed effects were independent of the $P2Y_{12}$ receptor as well as ENT1 indicating that other mechanisms may be involved.[1] Yet, specific additional target receptors of ticagrelor remain to be determined. To assess the physiological relevance of our *in vitro* data *in vivo*, we showed that ticagrelor, unlike clopidogrel, reduced endothelial TF expression in common carotid arteries and prolonged arterial occlusion times in mice; further, we ruled out other potential and relevant mechanisms, which could have contributed to this observation including platelet aggregation, plasma TF activity and systemic coagulation.[1] In order to finally prove the relevance of endothelial TF in our experiments, a rescue experiment specifically preventing the reduction of endothelial TF by ticagrelor *in vivo*, without affecting other sources of TF, appears essential.

Our study indicates local antithrombotic effects of ticagrelor at the vessel wall in addition to its antiplatelet effects.[1] Such properties may have contributed to the reduction of clinical events including MI and stroke in ACS patients treated with ticagrelor in clinical trials.[18,30] On the other hand, dual antithrombotic effects of ticagrelor may contribute to an increased bleeding risk. Although ticagrelor did not augment total major bleeding events, fatal intracranial bleeds were higher in ACS patients treated with ticagrelor, compared with clopidogrel.[18] Whether this observation may be due to higher platelet inhibition[23] or due to the local antithrombotic properties of ticagrelor[1] remain speculative.

In a subsequent study we investigated whether the antithrombotic properties displayed by ticagrelor[12] could also be relevant in AF patients. Previously it was reported that LAA endocardial cells express higher levels of procoagulant TF and PAI-1, as compared with right atrial appendages, which may contribute to the higher thrombogenicity observed in LAA.[13]

Therefore, we investigated whether ticagrelor or CAM may affect TF or PAI-1 expression in LAA endocardial cells isolated from AF patients. Indeed, we found that ticagrelor, but not CAM, reduced TF and PAI-1 protein expressions as well as enzyme activities indicating local antithrombotic mechanisms in AF patients.[12] AF is a common cardiac arrhythmia, which is associated with an increased risk of stroke and mortality and oral anticoagulation is the treatment of choice to reduce thromboembolic complication.[10] Indeed, anticoagulation has been shown to be superior to antiplatelet therapy (single or DAPT).[61] Yet, newer and more potent generations of $P2Y_{12}$ receptor antagonists including ticagrelor have not been compared to oral anticoagulation in AF patients in randomized clinical trials. The described local antithrombotic properties of ticagrelor in LAAs of AF patients may be of clinical relevance and may affect thrombogenicity in these patients;[12] however, this hypothesis needs to be addressed in additional clinical studies.

Frequently, AF patients have comorbidities such as ACS and require both anticoagulant and antiplatelet therapy, referred to as triple anticoagulation.[11] In such patients, ticagrelor may be the preferred choice in order to reduce thromboembolic complications. Triple anticoagulation on the other hand increases bleeding complications substantially and choosing the ideal treatment to prevent thrombotic events without causing bleedings is challenging.[11] In patients with an ACS receiving DAPT (aspirin and a thienopyridine), small dosages of oral anticoagulants are sufficient to increased bleeding rates significantly.[62] Consequently, in patients with anticoagulant treatment undergoing percutaneous intervention requiring DAPT, single (clopidogrel) versus DAPT reduced bleeding complications significantly;[63] however, although not statistical significant, higher rates of thrombotic events have been observed in the single antiplatelet therapy group.[63] Therefore, it remains to be determined whether reducing triple antithrombotic therapy to oral anticoagulation and $P2Y_{12}$ receptor inhibition is sufficient to sustain low thrombotic events. The local anti-thrombotic properties of ticagrelor appear beneficial in AF patients requiring platelet antagonists due to comorbidities such as

ACS; however, further clinical studies are required to prove the effectiveness and safety of newer generations of $P2Y_{12}$ receptor antagonists such as ticagrelor in AF.[64]

9 Abbreviations

ACS	acute coronary syndrome
ADP	adenosine diphosphate
AF	atrial fibrillation
CAM	clopidogrel active metabolite
CURE	Clopidogrel in Unstable Angina to Prevent Recurrent Events
CV	cardiovascular
CVD	cardiovascular disease
DAPT	dual antiplatelet therapy
eNOS	endothelial nitric oxide synthase
ENT1	equilibrative nucleoside transporter 1
GP	glycoprotein
H2O2	hydrogen peroxide
HAECs	human aortic endothelial cells
ISIS-2	second international study of infarct survival
LAA	left atrial appendage
LDL	low-density lipoprotein
MI	myocardial infarction
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
O^2-	superoxide anion
OH	hydroxyl radicals
ONOO-	peroxynitrite
PAI-1	plasminogen activator inhibitor-1
PGI_2	prostacyclin
PI3K	phosphoinositide 3-kinase
PLATO	study of Platelet Inhibition and Patient Outcomes

ROS	reactive oxygen species
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cells
vWF	von Willebrand factor

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11 Declaration of personal contributions to work

11.1 Ticagrelor, but not Clopidogrel, Reduces Arterial Thrombosis via Endothelial Tissue Factor Suppression

Cell culture experiments, western blotting, ELISA, data analysis and interpretation in Fig. 1 were done by myself.

Cell culture experiments, data analysis and interpretation in Fig. 2A and B were done by myself, rt-PCR was performed by Alexander Akhmedov, PhD.

Cell culture experiments, western blotting, data analysis and interpretation in Fig. 3A and B were done by myself.

Cell culture experiments, western blotting, data analysis and interpretation in Fig. 4A and B were done by myself, rt-PCR in Fig. 4A was performed by Alexander Akhmedov, PhD.

Cell culture experiments, western blotting, data analysis and interpretation in Fig. 5A and C-D were done by myself. Cell culture experiments, data analysis and interpretation in Fig. 5B were done by myself, rt-PCR was performed by Nicole Bonetti, MD.

Platelet isolation and aggregometry, data analysis and interpretation in Fig. 6A was done by myself. Measurement of ticagrelor plasma concentrations in Fig. 6B was performed by AstraZeneca, Mölndal, Sweden. Surgical procedure, data analysis and interpretation in Fig. 6C was performed by myself. Endothelial TF staining in Fig. 6D was performed by Sophistolab AG, Muttenz, Switzerland; data analysis and interpretation was done by myself. Plasma TF measurement by ELISA in Fig. 6E and endogenous thrombin potential in Fig. 6F as well data analysis and interpretation was done by myself.

Cell culture experiments, western blotting, data analysis and interpretation in Supplemental figure 1 were done by myself.

11.2 Ticagrelor, but not Clopidogrel Active Metabolite, Displays Antithrombotic Properties in the Left Atrial Endocardium

Endocardial cell isolation from atrial appendages of atrial fibrillation patients was performed by Heidi Amstalden, MSc and Martina Glanzmann MSc. Cell culture experiments, western blotting and data analysis in Fig. 1A, B, D and E was performed by Heidi Amstalden, MSc, Martina Glanzmann MSc and myself, data interpretation was done by myself. TF and PAI-1 activity in Fig. 1C and F, respectively, data analysis and interpretation was done by myself. Fig. 1 G was designed by PD Dr. med. Jan Steffel.

Cell culture experiments, western blotting, data analysis and interpretation in Supplemental figure 1 were done by myself.

12 Curriculum Vitae

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Personal information

Nationality	Austrian
Date of Birth	12 June 1986
Place of Birth	Innsbruck, Austria
Marital Status	Single

Current career

Resident at the Department of Internal Medicine, Cantonal Hospital Baden, Baden, Switzerland (12/2015 – to date) and

MD-PhD student at the Center for Molecular Cardiology, University of Zurich, Schlieren, Switzerland (07/2012 – to date)

Education and degrees

2014 – 2016	Dr. med. (M.D.), Medical University Zurich, Switzerland Dietary omega-3 alpha-linolenic acid does not prevent venous thrombosis in mice
2006 – 2012	Dr. med. univ. (M.D.), Medical University Innsbruck, Austria Cardiac Morphology and Function in Migfilin-Deficient Mice due to Experimental Pressure Overload
2005 – 2006	Military Service as Paramedic (8 months), Innsbruck, Austria
2000 – 2005	Commercial high school Innsbruck, Austria

Reviewer for scientific journals and institutions

European Heart Journal, Thrombosis Research, Nutrients, Frontiers Physiology, Molecules

Awards and prizes

04/2017 Best Thesis Award 2017, Medical University of Zurich, Switzerland

Dietary omega-3 alpha-linolenic acid does not prevent venous thrombosis in mice

06/2016 Best Abstract in Cardiovascular Biology, Swiss Society of Cardiology

Reiner MF, Diaz-Cañestro C, Akhmedov A, Amstalden H, Briand S, Semerano A, Giacalone G, Keller S, Kullak-Ublick GA, Sessa M, Lüscher TF, Beer JH, Camici GG. Silencing of the Activated Protein-1 Transcription Factor JunD Exacerbates Ischemia/Reperfusion-induced Cerebral Injury.

Cardiovascular Medicine 2016; 19 (Suppl 26)

08/2015 Travel Grant, European Society of Cardiology

06/2014 Young Investigator Award, 60th Annual Meeting of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis

Reiner MF, Stivala S, Lüscher TF, Camici GG, Xiu-Fen M, Yang Z, Beer JH. Arginase II KO reduces platelet aggregation while sparing coagulation in aged mice. *J Thromb Haemost. 2014, Suppl 1:1-106*

Invited lectures

05/2015 Viel Fett, viel Salz: Ist Käse gesund? (A lot of fat and salt: Is cheese healthy?)

Nutrition, Bregenz, Austria

09/2014 Cheese: impacting clinical outcomes by modulation of dietary lipids

European Society for Clinical Nutrition and Metabolism, Geneva, Switzerland

Grants

2017 47'500 CHF, Foundation Kardio Baden (Switzerland), co-applicant

2014 20’000 CHF, Hartmann Müller-Foundation for Medical Research, main applicant

Personal skills and language

German: first language

English: C2

French: A2

Zurich, 20 September 2017

List of publications

Original articles (O)

O13 Akhmedov A, Camici GG, **Reiner MF**, Bonetti N, Costantino S, Holy EW, Spescha RD, Stivala S, Schaub Clerigué A, Speer T, Breitenstein A, Manz J, Lohmann C, Paneni F, Beer JH, Lüscher TF.

Endothelial LOX-1 Activation Differentially Regulates Arterial Thrombus Formation Depending on oxLDL Levels: Role of the Oct-1/SIRT1 and ERK1/2 Pathways.

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O12 **Reiner MF**, Breitenstein A, Holy EW, Glanzmann M, Amstalden H, Stämpfli SF, Bonetti NR, Falk V, Keller S, Savarese G, Benussi S, Maisano F, Lüscher TF, Beer JH, Steffel J, Camici GG.

Ticagrelor, but not clopidogrel active metabolite, displays antithrombotic properties in the left atrial endocardium.

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O11 **Reiner MF**, Akhmedov A, Stivala S, Keller S, Gaul DS, Bonetti NR, Savarese G, Glanzmann M, Zhu C, Ruf W, Yang Z, Matter CM, Lüscher TF, Camici GG, Beer JH. Ticagrelor, but not clopidogrel, reduces arterial thrombosis via endothelial tissue factor suppression.

Cardiovasc Res. 2017 Jan;113(1):61-69.

O10 **Reiner MF**, Stivala S, Limacher A, Bonetti NR, Méan M, Egloff M, Rodondi N, Aujesky D, von Schacky C, Lüscher TF, Camici GG, Beer JH.

Omega-3 Fatty Acids Predict Recurrent Venous Thromboembolism or Total Mortality in Elderly Patients with Acute Venous Thromboembolism.

J Thromb Haemost. 2017 Jan;15(1):47-56.

O9 Breitenstein A, Stämpfli SF, **Reiner MF**, Shi Y, Keller S, Akhmedov A, Schaub Clerigué A, Spescha RD, Beer HJ, Lüscher TF, Tanner FC, Camici GG.

The MAP kinase JNK2 mediates cigarette smoke-induced arterial thrombosis.

Thromb Haemost. 2017 Jan 5;117(1):83-89.

O8 Spescha RD, Klohs J, Semerano A, Giacalone G, Derungs RS, **Reiner MF**, Rodriguez Gutierrez D, Mendez-Carmona N, Glanzmann M, Savarese G, Kränkel N, Akhmedov A, Keller S,

Mocharla P, Kaufmann MR, Wenger RH, Vogel J, Kulic L, Nitsch RM, Beer JH, Peruzzotti-Jametti L, Sessa M, Lüscher TF, Camici GG.

Post-ischaemic silencing of p66Shc reduces ischaemia/reperfusion brain injury and its expression correlates to clinical outcome in stroke.

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O7 Haubner BJ, Moik D, Schuetz T, **Reiner MF**, Voelkl JG, Streil K, Bader K, Zhao L, Scheu C, Mair J, Pachinger O, Metzler B.

In Vivo Cardiac Role of Migfilin during Experimental Pressure Overload.

Cardiovasc Res. 2015 Jun 1;106(3):398-407.

O6 Savarese G, Rosano GM, Parente A, D'Amore C, **Reiner MF**, Camici GG, Trimarco B, Perrone-Filardi P.

Reduction of C-reactive protein is not associated with reduced cardiovascular risk and mortality in patients treated with statins. A meta-analysis of 22 randomized trials.

Int J Cardiol. *2014 Nov 15;177(1):152-160.*

O5 Akhmedov A, Montecucco F, Braunersreuther V, Camici GG, Jakob P, **Reiner MF**, Glanzmann M, Burger F, Paneni F, Galan K, Pelli G, Vuilleumier N, Belin A, Vallée JP, Mach F, Lüscher TF.

Genetic deletion of the adaptor protein p66Shc increases susceptibility to short-term ischaemic myocardial injury via intracellular salvage pathways.

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O4 **Reiner MF**, Martinod K, Stivala S, Savarese G, Camici GG, Lüscher TF, Wagner DD, Beer JH.

Dietary omega-3 alpha-linolenic acid does not prevent venous thrombosis in mice.

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O3 Holy EW, Besler C, **Reiner MF**, Camici GG, Manz J, Beer JH, Lüscher TF, Landmesser U, Tanner FC.

High-density lipoprotein from patients with coronary heart disease loses anti-thrombotic effects on endothelial cells: impact on arterial thrombus formation.

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O2 Savarese G, Dei Cas A, Rosano G, D'Amore C, Musella F, Mosca S, **Reiner MF**, Marchioli R, Trimarco B, Perrone-Filardi P.

Reduction of albumin urinary excretion is associated with reduced cardiovascular events in hypertensive and/or diabetic patients. A meta-regression analysis of 32 randomized trials.

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O1 Stivala S, **Reiner MF**, Lohmann C, Lüscher TF, Matter CM, Beer JH.

Dietary α-linolenic acid increases the platelet count in ApoE-/- mice by reducing clearance.

Blood. 2013 Aug 8;122(6):1026-33.

Reviews (R)

R3 Reiner MF, Stivala S, Beer JH.

Alpine cheese in cardiovascular disease.

Eur Heart J. 2015 Aug 14;36(31):2023-2030. CardioPulse Articles.

R2 Reiner MF, Stivala S, Camici GG, Beer JH.

The effects of Omega-3 fatty acids in clinical medicine.

Praxis (Bern 1994). 2014 Mar 12;103(6):329-35.

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Omega-3-Fettsäuren, Schweizer Alpkäse und deren Auswirkungen auf das kardiovaskuläre System.

Schweiz. Zeitschr. f. Ernährungsmed. 2012;5:1–5.